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<td>David, Brit</td>
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Osaka University
Substrate and nucleus connections to actin filament mediate embryonic stem cell differentiation

Graduate School of Frontier Biosciences
Osaka University

Brit Gracy David
One held my hand and lifted me up when I fell down,
Another held my hand pertinaciously at all times
And the little one, daintily embraced me and led me onward
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Abstract

Embryonic stem cells (ESCs) are pluripotent cells with the capabilities to self-renew and differentiate into any cell type in the body, and these remarkable abilities promise wide-ranging therapeutic applications. Mechanism of pluripotency of the stem cells has been investigated by the regulation of the three core transcription factors, Oct4, Nanog and Sox2. Here, I have instead investigated the mechanism of the pluripotency maintenance of ESCs from the view of mechano-sensing. In this study, I found that mouse ESCs spontaneous differentiation can be attenuated using polyacrylamide gels as soft substrates: ESCs on soft substrates continued to form round and compact colonies, with high alkaline phosphatase (AP) activity, a marker for pluripotency activity, and with high expression levels of Oct4, Nanog and Sox2, even in the absence of leukaemia inhibitory factor (LIF), which maintains the stable expression of these factors. In these cells, both talin and alpha catenin structures were absent and actin filaments (F-actin) were disrupted, indicating that the transmission of mechanical forces from the substrates trigger downstream signaling cascades that lead to a force-dependent change in the expression of the pluripotent genes. Moreover, disrupting Nesprin-mediated connection between F-actin and the nucleus through overexpression of the dominant negative KASH (Klarsicht/ANC-1/Syne-1 homology) domain of Nesprin 1 and Nesprin 2 showed high expression of Oct4, Nanog and Sox2 levels even in the absence of LIF at day 4 of culture. In conclusion, pluripotency of ESCs can be sustained through mechano-stress mediated by Nesprin, a protein linking F-actin and nuclear membrane, and is controllable by external mechanical perturbation such as elasticity of the substrate.
Chapter 1 General Introduction

1.1 Introduction to stem cells

Embryonic stem cells (ESC) can aid functional regenerative medicine because of their abilities to self-renew and differentiate to any cell type in the body. Stem cells can be segregated into two distinct states; the naïve ground state and the primed state (Nichols and Smith, 2009). ESCs in the naïve ground state are derived from the embryonic inner cell mass (ICM) of a mouse blastocyst at day 4-5 of embryonic development (Martin, 1981; Evans and Kaufman, 1981). Cells in the primed state are obtained from the epiblast of post-implantation mouse embryos and are called epiblast stem cells (EpiSC) (Gardner, 1998). As shown in Table 1-1, the two states differ in colony morphology, growth factors needed for the cells’ proliferative maintenance, single cell renewal capability, and the regulation of the transcription factors (Nichols and Smith, 2009; Weinberger et al., 2016). The pluripotency of ESC is regulated by gene regulatory networks that include the core transcription factors Oct4, Nanog and Sox2. Human ESCs have been isolated previously (Thomson, 1998; Gearhat, 1998), however, it sparked ethical controversy due to the use of human embryos in the process. However, in 2006, a major breakthrough in stem cell research and regenerative medicine occurred with the generation of induced pluripotent stem cells (iPSC) from somatic cells, which excludes the usage of human embryos (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). The somatic cells were reprogrammed with the expression of transcription factors Oct4, Sox2, Klf4 and cMyc. The breakthrough has prompted scientists to try various methods to generate iPSCs with simpler techniques and better yields (Okita et al., 2011; Zhou et al., 2009; Stadtfeld et al., 2010). Figure 1-1 illustrates ESC isolation
from the blastocyst and the stable ESC cell line cultivation in vitro. The availability of such cell lines helps investigators to reveal the characteristics and capabilities of pluripotent cells for regenerative medicine applications.

**Table 1-1** Comparison of stem cells characteristics between the ground state ESC and the primed state EpiSC

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Ground state</th>
<th>Primed state</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Embryonic body formation</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Single cell renewal</strong></td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td><strong>Growth in feeder-free culture</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Colony morphology</strong></td>
<td>Domed</td>
<td>Flattened</td>
</tr>
<tr>
<td><strong>Transcription factors</strong></td>
<td>Oct4, Sox2, Nanog, Klf2, Klf4</td>
<td>Oct4, Sox2, Nanog</td>
</tr>
<tr>
<td><strong>Regulation of external signaling pathways</strong></td>
<td>LIF/STAT3, BMP4, WNT, IGF</td>
<td>TGF-β, Activin, FGF2, ERK1/2, WNT, IGF</td>
</tr>
</tbody>
</table>
Figure 1-1 Illustration of ESC derivation from ICM and its cultivation in the laboratory for possible regenerative medicine applications
1.2 What defines the pluripotency of ESCs?

1.2.1 Pluripotency assays

The pluripotency of stem cells can be assessed in several ways: (1) the expression of core transcription factors, namely Oct4, Nanog and Sox2, which are silent in differentiated somatic cells; (2) the formation of teratocarcinoma when the pluripotent cells are injected subcutaneously in mice; (3) the ability to differentiate and initiate all cell lineages of an organism, particularly the formation of the three germ layers, endoderm, ectoderm and mesoderm, from which different tissue types develop; and (4) the formation of chimera, demonstrating the presence of pluripotent cells that were injected in mouse embryos and proving that they have matured into cells of germline (Martin and Evans, 1975; Smith et al., 2001).

1.2.2 Gene regulatory network

Maintaining the pluripotency of ESC is an in vitro procedure that enables the cells to self-renew indefinitely and differentiate into all cell types. To retain the self-renewal capability of ESCs in the laboratory, the culture environment should provide the necessary nutrients and signals, and inhibit the ability of ESC to differentiate spontaneously. (Babaie et al., 2007; Mitsui et al., 2003; Loh et al., 2006). Various studies have established Oct4, Sox2 and Nanog as the core transcription factors that regulate the pluripotency of ESCs (Yuan et al., 1995; Nicholas et al., 1998; Niwa et al., 1998; Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003; Masui et al., 2007). Oct4 is recognized as the key regulator of the pluripotency of ESCs (Nichols et al., 1998; Boyer et al., 2005), and it has also been reported to form a complex with Sox2 to maintain the pluripotency of ESCs (Chambers and Smith, 2004). Although the gene expression level of this complex is auto-
regulated (Chew et al., 2005), the complex itself enhances and is enhanced by the transcription factor Nanog, thus creating a positive feedback loop that maintains the pluripotency of the cells (Pan et al., 2006). Furthermore, these core transcription factors activate other genes specific to the maintenance of ESCs and further promote the undifferentiated state of ESCs (Avilion et al., 2003, Niwa et al., 2005, Pan et al., 2006; Masui et al., 2007; Chen et al., 2008; Chambers and Thomlinson, 2009).

Cells are defined as differentiating when the levels of the core transcription factors Oct4 and Nanog drop below a certain threshold (Osorno et al. 2012). Therefore, to sustain the pluripotency of ESCs, a significant expression level of these core transcription factors is required (Nichols et al, 1998; Boyer et al., 2005; Loh et al., 2006; Marson et al., 2008). These factors are influenced by signals from extracellular environment (Guenther, 2011) and form a transcriptional module that maintains the pluripotency of ESCs (Figure 1-2).

1.2.3 Sustaining the pluripotency of ESC in vitro

As described above, the regulation of core transcription factors Oct4, Nanog and Sox2 is important in the maintenance of ESC pluripotency in vitro. In addition, the signaling pathways illustrated in Figure 1-2 and Table 1-1 also play important roles in regulating the pluripotency of ESC in vitro. Conventionally, ESCs are sustained in culture media containing serum and exogenous leukemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988). LIF maintains the pluripotency of mouse cells via the JAK/STAT3 signaling pathway shown in Figure 1-2 (Ying et al., 2008, Niwa et al., 2009). LIF binds to its receptor, LIF receptor (LIFR) and a glycoprotein, GP130 that activates Janus kinase (JAK). Subsequently, JAK activates signal transducers and activators of
transcription (STAT3) (Stahl et al., 1995; Niwa et al., 1998; Auernhammer and Melmed, 2000). STAT3 then activates the transcription factor Klf4, which enhances Sox2. It was also reported that LIF triggers phosphatidylinositol 3-kinase (PI3K) signaling that activates T-box transcription factor (Tbx3), which subsequently activates Nanog (Niwa et al., 2009). Since these factors support the maintenance of pluripotency of ESCs, withdrawal of LIF from the cell culture causes the suppression of STAT3, and the eventual loss pluripotency (Niwa et al., 1998).

**Figure 1-2** Gene regulatory network that maintain the pluripotency of ESCs
1.3 Objective of this study

Although a large number of ESC pluripotency studies have focused on the biochemical regulation of gene regulatory network, recent works are also reporting the importance of mechanical signals from microenvironment in regulating the stem cell fate (Farge, 2011; Mamamoto and Ingber, 2010; Wozniak and Chen, 2009). Stem cells can undergo force-dependent change by a process called mechanotransduction, where extracellular mechanical signals that reach the cells are transduced into the nucleus and alter the fate of the cells. Mechanical signal arising from the stiffness of substrate is thought to play an important role in pluripotency regulation because unlike the condition of inner cell mass, *in vitro* culture on rigid dishes would generate different mechanical signals compared to the softer *in vivo* conditions. Yet most studies have focused on mechanical signals generated from cell-cell contacts of ESCs (Pieters and Van Roy, 2014; Sun et al., 2012), which is mediated by E-cadherin (Redmer et al., 2011). On the other hand, integrin-mediated focal adhesion, which is the sensory element that connects the cell membrane to the cell matrix, is responsible for the substrate stimuli (Krebsbach et al., 2017). Higuchi and colleagues recently reported that the inhibition of integrin signal by echistatin forms round and compact colonies with high Nanog expression (Higuchi et al., 2016). Studies on cell response to substrate mechanical properties have often focused on actin-related cytoskeletal components (Hou et al., 2013; Talwar et al., 2014). In addition to actin filaments, the plasticity of the nuclear membrane, which is regulated by the nuclear lamina, also governs the gene expressions in the pluripotent state (Pajerowski et al., 2007). Constantinescu and coworkers reported that at the early stage of ESC differentiation, nuclear stiffness increases and the condensation of chromatin in the stiff nucleus results in the down regulation of Nanog (Constantinescu et al., 2006). Despite
these important studies on mechanical regulation of ESC pluripotency, the mechanism of signal transmission from focal adhesion to the nucleus, which ultimately results in the regulation of gene expressions that govern pluripotency, is largely unexplored. Therefore, this dissertation focuses on focal adhesion and the physical link that connects it to the nuclear membrane through actin filaments, and describes the mechanism of mechano-sensing from the substrate to the expression of transcription factors in the maintenance of ESC pluripotency.
1.4 Thesis outline

This dissertation is divided into four chapters beginning with the general introduction (Chapter 1), which describes the background and motivations of this study. In Chapter 2, I investigated the effects of mechanical signals originating from substrate elasticity, on sustaining the pluripotency of mouse ESCs. Using polyacrylamide gels with varying Young’s moduli as substrates, I present the changes that take places in terms of cell morphology, organization of cytoskeletal proteins and the pluripotency of ESCs, when cultured with different substrate elasticities. The chapter shows that spontaneous differentiation of ESCs could be suppressed without LIF when cultured on soft substrates. High levels of pluripotency markers such as Oct4, Nanog and Sox2 were detected in cells cultured on soft substrate without LIF. Strikingly, ESCs on soft substrates have less developed cytoskeleton structures compared to those cultured on rigid substrates. When cultured on soft substrates in the absence of LIF, the lack of mechanical stimuli from the substrates plays a crucial role in the maintenance of pluripotency.

In Chapter 3, following the findings in Chapter 2, which showed that the mechanical signal from the substrate alters ESC actin cytoskeletal network according to the substrate rigidity, I hypothesized that such change in the cytoskeletal network would be sufficient to modify nuclear shape and gene expressions, and consequently regulate ESC pluripotency. Since nuclear membrane-linked Nesprin 1 and Nesprin 2 interact with cytoplasmic actin filaments, I show that disrupting their Klarsicht/ANC-1/Syne-1 homology (KASH) domain connection with F-actin, suppresses signal transmission from the substrate to the nucleus and ultimately alters the gene expressions of Oct4, Nanog and Sox2 in the absence of LIF.
Finally, in Chapter 4, I provide general discussions about the main findings in this dissertation and potential future directions of research.
Chapter 2 Regulating pluripotency maintenance of embryonic stem cells through substrate elasticity

2.1 Introduction

The distinctive characteristics of ESCs are its remarkable abilities to self-renew and differentiate into all cells of the body. These characteristics make them fit for use in functional tissue engineering and cell-based therapies. Pluripotency of ESCs is regulated by the dynamics of the core transcription factors, Oct4, Nanog and Sox2. However, pluripotent cells often display heterogeneous gene expression in vitro, resulting in spontaneous differentiation of the cells (Mummery et al., 1990; Nishikawa et al., 2007). Therefore, sustaining the pluripotency of these cells in the laboratory can be challenging. Efforts have been channeled over the years into discovering methods to sustain the pluripotency of ESCs triggered by the gene regulatory network (Smith et al., 1988; Williams et al., 1988; Sato et al., 2004; Yoshida et al., 1994; Niwa et al., 1998; Ying et al., 2008). Conventionally, to inhibit the spontaneous differentiation of mouse ESCs, chemical stimulation with the addition of leukemia inhibitory factor (LIF) can sustain the pluripotency of ESC which can be tested with the expression of Oct4, Nanog and Sox2 (Smith et al., 1988; Williams et al., 1988).

Apart from the chemical stimulation, stem cells are also influenced by the mechanical stimulation from the cell microenvironment but it is usually neglected in cell culture. The microenvironment’s mechanical signals are thought to be crucial for preserving cell pluripotency because cells in vivo may generate different signals compared to the ESCs culture in glass or plastic dishes. Stiffness of culture substrates where the cells were cultured onto, can influence stem cell phenotypes of
self-renewal and differentiation through the regulation of gene transcription and signaling pathways (Ahmed et al., 2016). Recently, synthetic gels that mimic the elasticity of the microenvironment in vivo, and infused with extracellular matrix (ECM) ligands, began to be used for studies of the impact of substrate elasticity on cell fate (Engler et al., 2006; Nawa et al., 2012). In vivo, the elasticity of the microenvironment is determined by the adherent cells and ECM’s properties. Elasticity is measured using the Young’s modulus, \( E \), which is defined as the resistance of a solid to mechanical stress. Brain tissues have an elasticity of \( E \approx 0.5 \text{ kPa} \), which is softer than muscle tissues \( (E \approx 10-20 \text{ kPa}) \). Muscle tissues, on the other hand, are softer than skin tissues \( (E \approx 50 \text{ kPa}) \). Recent studies used polyacrylamide gels with adjustable \( E \) (from soft to rigid) to be able to more closely match the elasticity of in vivo microenvironment (Engler et al., 2006). These studies revealed that ECM and substrate elasticity can regulate cell fate (Chowdury et al., 2010; Shimizu et al., 2012; Lv et al., 2015).

In general, when the substrate elasticity matches the stiffness of a specific tissue, stem cell differentiation is guided towards these specific tissue lineages. Substrates with the elastic moduli of brain \( (1 \text{ kPa}) \), muscle \( (8-17 \text{ kPa}) \) and bone \( (25-40 \text{ kPa}) \) direct mesenchymal stem cell differentiation into neurocytes, myoblasts and osteoblasts, respectively (Engler et al., 2006). Over the years, studies have also revealed the importance of substrate elasticity in controlling cell functions such as proliferation, migration and organization (Lo et al., 2000; Wang et al., 2000; Krieg et al., 2008; Chowdury et al., 2010). In our laboratory, mouse embryonic fibroblasts (MEFs) were successfully reprogrammed into partial induced pluripotent stem cells (iPSCs) using soft substrate elasticity (Higuchi et al., 2014). Therefore, the roles of ECM and substrate in determining cell fate and functions need to be considered when sustaining the pluripotency of
ESCs in vitro. In the beginning of this work, I investigated the adaptation mechanisms of mouse ESCs cultured on soft substrates because studies on ESCs and mechanisms of their pluripotency maintenance through mechanical regulation is still scarce. In this chapter I present my work on the influence of polyacrylamide gel substrates with varying Young’s moduli on the properties of mouse ESCs related to cell fate.
2.2 Materials and methods

2.2.1 Cell culture

Cell line E14Tg2a of mouse embryonic stem cells was used in this study and they were routinely cultured on plastic culture dishes (Falcon; BD Biosciences, Franklin Lakes, NJ; catalog numbers 353003, 353001, 353046 and 353004) coated with 0.1% gelatin (Sigma-Aldrich, MO, USA). The cells were incubated at 37°C in an atmosphere of 5% CO₂ supplemented with Dulbecco’s Modified Eagle’s Medium (DMEM, D6046; Sigma-Aldrich, MO, USA) containing 10% fetal bovine serum (FBS; ThermoFisher Scientific, MA, USA) 1% penicillin antibiotics (Sigma-Aldrich, MO, USA), 1% streptomycin (Sigma-Aldrich, MO, USA), 1% Gibco GlutaMAX-I L-glutamine (ThermoFisher Scientific, MA, USA), 1% minimum essential medium (MEM) non-essential amino acids (ThermoFisher Scientific, MA, USA), 1% nucleosides (Sigma-Aldrich, MO, USA), 1% sodium pyruvate (Sigma-Aldrich, MO, USA), 0.1% 2-mercaptoethanol (Sigma-Aldrich, MO, USA) and 0.1% leukemia inhibitory factor (LIF; NACALAI TESQUE, INC., Japan). Media were changed every two days and cells were harvested when they are 70% confluent. For the experiments with the polyacrylamide gel, cell cultures were performed on 0.1% gelatin (Sigma-Aldrich, MO, USA) coated 25-mm glass coverslip (Fisher Scientific, Germany) without polyacrylamide gel coating to represent rigid substrates (50 GPa); and as for the soft and intermediate substrates, cell cultures were performed on 0.1% gelatin (Sigma-Aldrich, MO, USA) coated 25-mm glass coverslips (Fisher Scientific, Germany) covered with polyacrylamide gels of 1 kPa and 10 kPa Young’s modulus (E), respectively. The cells were cultured up to 4 days in culture media containing LIF to maintain the
undifferentiated state of the mouse ESCs. To observe the spontaneous differentiation of the cells, ESCs were cultured in culture media with the absence of LIF for 4 days (unless stated otherwise).

2.2.2 Preparation of polyacrylamide gels

Polyacrylamide gels of 1 kPa and 10 kPa Young’s modulus (E) was prepared as previously reported (Higuchi et al., 2014) with slight modifications. The preparation steps are illustrated in Figure 2-1. Clean 25-mm circular glass coverslips (Fisher Scientific, Germany) that have been washed with 100% ethanol were first soaked in silane-coupling agent (3-methacryloxypropyltrimethoxysilane; Shin-Etsu Chemical Co., Ltd, Japan; catalog number KBE-503) for 1 hour to ease attachment of the polyacrylamide gel onto the glass coverslip surface. Next, the silane-coated glass coverslips were washed with ethanol again and dried. Subsequently, acrylamide (Sigma-Aldrich, MO, USA) and methylenebisacrylamide (Sigma-Aldrich, MO, USA) solution in distilled H₂O prepared to the concentrations as listed in Table 2-1 was vortexed with 1 µl of Tetramethylethylenediamine (TEMED; Bio-Rad, Hercules, CA, USA) and 10 µl of ammonium persulfate (APS; Sigma-Aldrich, MO, USA) to make polyacrylamide gels of 1 kPa and 10 kPa respectively. Then, 8.5 µl drop of the polyacrylamide solution was carefully placed on top of the silane-coated glass coverslip. Afterwards, a clean 22-mm glass coverslip (Fisher Scientific, Germany) that has been washed with 100% ethanol and dried was carefully placed on top of the 25-mm glass coverslip containing the 8.5 µl polyacrylamide solution. This procedure was done to sandwich and to spread the polyacrylamide solution on the 25-mm glass coverslip. The sandwiched solution was subsequently incubated at room temperature approximately for 15 min to allow polymerization of the gels. It is important to note that 1 kPa solution which makes softer substrates than 10 kPa takes longer to solidify. After confirming that the gels have polymerized, the top 22-mm glass
coverslip was discarded very carefully leaving the polymerized gel on the 25-mm glass coverslip. The polyacrylamide gel coated 25-mm glass coverslips were immediately immersed in 50 mM HEPES buffer (pH 8.5) and left in the shaker for 1 h at room temperature to remove the excess unpolymerized acrylamide. Next, the polyacrylamide gels were coated with an intermediate coupling agent to allow attachment of gelatin onto the polyacrylamide gel. Approximately 300 µl of 0.05% Sulfosuccinimidyl-6-(4’-azido-2’-nitrophenylamino) hexanoate (Sulfo-SANPAH; Thermo Fisher Scientific, MA, USA) solution was added to cover the polyacrylamide gel surface on the 25-mm glass coverslips. The coverslips were then irradiated in 312 nm ultra-violet (UV) light source at a distance of 10 cm for 30 min until the color of the Sulfo-SANPAH turns to rust brown. Photoactivation at 312 nm, allows phenylazide group of Sulfo-SANPAH to covalently attach to the gel and freeing succinimidyl ester to react with the amino groups of gelatin. Polyacrylamide gel coated glass coverslips were then rinsed with 50 mM HEPES buffer (pH 8.5) thrice and left in the shaker for 1 h to eliminate excess Sulfo-SANPAH. Then the glass coverslips with the gel coatings of 1 kPa and 10 kPa were sterilized by UV under the hood for 10 min before finally soaking them in 50 mM HEPES buffer (pH 8.5) containing 0.1% gelatin (Sigma-Aldrich, MO, USA). Both the gelatin coated polyacrylamide gels and the glass coverslips without polyacrylamide gel coating (representing rigid substrate) were incubated overnight at 37°C in an atmosphere of 5% CO₂. The next morning, the gelatin was aspirated and the polyacrylamide gels and the glass coverslips were dried in the incubator for 1 h before cell culture.
Table 2-1 Ratio of acrylamide to methylenebisacrylamide solution to make polyacrylamide gels with Young’s modulus, 1 kPa and 10 kPa

<table>
<thead>
<tr>
<th>Elasticity (kPa)</th>
<th>Acrylamide %</th>
<th>Bis-acrylamide %</th>
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<tr>
<td>1</td>
<td>5</td>
<td>0.03</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>0.1</td>
</tr>
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</table>
Figure 2-1 Schematic illustration on the protocol to coat polyacrylamide gel on the glass coverslips

Circular glass coverslips of 22 mm and 25 mm were washed with ethanol and dried.

25 mm glass coated with silane coupling reagent.

8.5 µl of acrylamide/bisacrylamide mixture with TEMED and APS was dropped.

22 mm glass was placed on top to sandwich the mixture.

25 mm glass coated with substrate of adjustable elasticity.

Covalently link sulfo-SANPAH to PA gel using UV light.

Wash off excess sulfo-SANPAH.

Link the ECM protein to sulfo-SANPAH.

Wash off excess ECM protein.
2.2.3 Quantification of gene expression

Gene expression levels of the undifferentiated ESCs cultured in the presence of LIF as well as the differentiated ESCs cultured in the absence of LIF for 4 days on 1 kPa, 10 kPa and glass substrates were quantified by quantitative real-time polymerase chain reaction (qRT-PCR). Firstly, RNA of the differentiated and the undifferentiated ESCs was isolated using RNeasy mini kit (Qiagen, Germany) and converted into cDNA using Omniscript RT Kit (Qiagen, Germany) according to manufacturer’s instructions. The cDNA was analyzed using primers conforming to *Mus Musculus* sequences as listed in Table 2-2 for qRT-PCR. To perform the analyses, a mixture of Thunderbird SYBR qPCR mix (TOYOBO, Japan), gene-specific primers (0.4 µM), cDNA template (300 ng) and distilled water were prepared, making a total of 8 µL mixture for each well. The mixture was reacted in a CFX96 real-time PCR analysis system (Bio-Rad, Hercules, CA, USA) with each sample performed in triplicate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. The qRT-PCR conditions used for the experiments were: 98°C for 30 s, 33 cycles of 98°C for 5 s, 54.5°C (Nanog), 57.3°C (Oct4), 54.6°C (Sox2), 53.4°C (GAPDH), 55°C (Lin28, Rex1, Eras, Klf4, Fgf4, Stat3, RhoA, ROCK1 and ROCK2) for 30 s respectively, 50°C for 5 s and 95°C for 5 s. Expression of Oct4, Nanog, Sox2, Lin28, Rex1, Eras, Klf4, Fgf4, Stat3, RhoA, ROCK1 and ROCK2 proteins at the mRNA level was normalized to GAPDH, which showed very little variation between the undifferentiated and differentiated ESCs.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5’-GCACAGTCAAGGCCGAGAAT-3’</td>
<td>5’-GCCCTTCCATGGGTGGTGA-3’</td>
</tr>
<tr>
<td>Oct4</td>
<td>5’-TCTTTCCACCAGGCCCGGCTC-3’</td>
<td>5’-TGCGGGCCGACATGGGGAGATCC-3’</td>
</tr>
<tr>
<td>Nanog</td>
<td>5’-AGGGTCTGCTACTGAGATGCTCTG-3’</td>
<td>5’-CAACCACTGGTTTTTCTGACCACGG-3’</td>
</tr>
<tr>
<td>Sox2</td>
<td>5’-TAGAGCTAGCCGCGGCTGATGA-3’</td>
<td>5’-TTGCTTAACAAGACCACAGGAA-3’</td>
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<tr>
<td>Lin28</td>
<td>5’-CCAATGTAATCTGTACCCCTTAG-3’</td>
<td>5’-GCTTTGTTTCTAGCAAGAACA -3’</td>
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<tr>
<td>Rex1</td>
<td>5’-ATTCCATGGTAGGTTCAACAGG-3’</td>
<td>5’-TGTGACTACTGCCAAGATTGGCC -3’</td>
</tr>
<tr>
<td>Eras</td>
<td>5’-TGTGGCGTCTGCTGAGCTGAA-3’</td>
<td>5’-TCATAAACACCTAGCAATGAGG -3’</td>
</tr>
<tr>
<td>Klf4</td>
<td>5’-AGTTTCCCAACTGCTGACT-3’</td>
<td>5’-AACACATTGTGCTGTTAA-3’</td>
</tr>
<tr>
<td>Fgf4</td>
<td>5’-AAGACGCTTCATAGGCCCAGG-3’</td>
<td>5’-AGCAGTTAGGCCAGCCTGGCT-3’</td>
</tr>
<tr>
<td>Stat3</td>
<td>5’-CTTGCTACTCTCTACCCG-3’</td>
<td>5’-GATCCATGTCAACCGTGAGCG-3’</td>
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<tr>
<td>RhoA</td>
<td>5’-TTCGGAATGACGACACACAG-3’</td>
<td>5’-GTCTAGCTTGCAHACGCT-3’</td>
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<tr>
<td>Rock1</td>
<td>5’-TTCATGTCGGACCTGAAACC-3’</td>
<td>5’-TTGAGCAGCGTGGAGGAGG-3’</td>
</tr>
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<td>Rock2</td>
<td>5’-TTCACGTCGGACCTGTTACC-3’</td>
<td>5’-GTGGCACCTAGCCGACCTCTA-3’</td>
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</table>
2.2.4 Immunofluorescence

The sources of primary antibodies used in the experiments were Oct4 mouse monoclonal antibody (sc-5279, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Sox2 rabbit polyclonal antibody (ab97959, Abcam, UK), Nanog goat polyclonal antibody (sc-30328, Santa Cruz Biotechnology), α-E-catenin mouse monoclonal antibody (sc-9988, Santa Cruz Biotechnology), and anti-Talin mouse monoclonal antibody (T3287, Sigma-Aldrich, MO, USA).

Generally, ESCs were cultured on substrates of 1 kPa and 10 kPa elasticity as well as glass for 4 days in the presence of LIF in the culture media to sustain the pluripotency of the cells and in the absence of LIF to accommodate spontaneous differentiation of the cells. After 4 days, the cells were washed with 1 × phosphate-buffered saline (PBS; NACALAI TESQUE, INC., Japan) once before fixing with 4% paraformaldehyde (PFA; EMS, Hatfield, PA, USA) for 5 min at room temperature. Fixed cells were then washed with 1 × PBS thrice for 5 min each and incubated in 0.01% TWEEN®20 (Sigma-Aldrich, MO, USA) in PBS for 5 min. The samples were subsequently blocked with blocking agent, CAS-Block (Thermo Fisher Scientific, MA, USA) solution for 30 min at room temperature. Primary antibodies were diluted at 1:100 with CAS-Block blocking agent and incubated either for 1 h at room temperature or overnight at 4°C. After 1 h or the next morning, the cells were washed with 1 × PBS thrice for 5 min each. The fluorescent secondary antibodies used in the experiment were Invitrogen anti-mouse Alexa Fluor 488 IgG (Thermo Fisher Scientific, MA, USA), Invitrogen anti-rabbit Alexa Fluor 594 IgG (Thermo Fisher Scientific, MA, USA) and Invitrogen anti-goat Alexa Fluor 647 IgG (Thermo Fisher Scientific, MA, USA) diluted at 1:400 with CAS-Block blocking agent and the samples were stained for 1 h at room temperature. Mouse ESCs were stained with Invitrogen Phalloidin-Alexa Fluor 594 to label F-
actin. Finally, the samples were rinsed with 1 × PBS thrice for 5 min each before imaging. Samples were examined with an Olympus FV 1000 (Olympus, Hamilton, Bermuda) laser scanning confocal microscope with 60× 1.4NA oil immersion objective.

2.2.5 Image analysis

Images were saved as original imaging format (.oif) files with dimensions of 2048 × 2048 pixels and a file depth of 16 bits. All image analyses were performed using ImageJ software (version 1.47). Fluorescent intensities were measured by quantifying the mean pixel intensities normalized with cell area of cells selected from random colonies for each culture conditions. To obtain the co-localization of F-actin to Talin and F-actin to alpha-catenin to evaluate how well the two respective proteins correlate in their intensities, scatter plots of bright pixels of one channel that coincide with the bright pixel of another channel were schemed. Cell area and pixel intensities were quantified from at least 50 cells from random colonies for each culture conditions.

2.2.6 Statistical analyses

Data presented in this work were obtained from at least three independent experiments and presented as means with standard deviations (SD). Student’s t-test was performed to calculate the statistical significance among data sets with values of \( p < 0.05 \) considered significant.
2.3 Results

2.3.1 Adaptation of mouse ESCs to various substrate elasticity

Using gelatin coated glass coverslips of 50 GPa Young’s modulus and polyacrylamide gels of 1 kPa and 10 kPa, I first investigated the ability of mouse ESCs to adapt to the elasticity of their surrounding substrates. To maintain the biochemical appearance of extracellular matrix (ECM) ligands, 1 kPa and 10 kPa substrates and glass substrates were coated with 0.1% gelatin. To exclude the possible influence of external signals and observe cells in standard culture conditions, I removed LIF from the culture of mouse ESCs on 1 kPa, 10 kPa and glass substrates. As a control and to maintain the standard culture conditions, three samples of cells were cultured on corresponding substrates in media containing LIF. The cells were left to proliferate for 4 days, and changes in cell morphology became obvious at day 4. Under the standard culture conditions with the addition of LIF, control ESCs on soft substrates (1 kPa) formed round and compact colonies as shown in Figure 2-2. Similar results were observed in control ESCs cultured on the 10 kPa and the rigid glass substrates. Interestingly, ESCs cultured on 1 kPa and 10 kPa without LIF formed round and compact colonies similar to the control cells with LIF. However, on rigid glass dishes, the culture without LIF produced irregularly shaped and widespread colonies. The spread morphology of the colonies on the glass substrate was distinct from the tight and round colonies on the soft and intermediate substrates. These results showed that in the absence of LIF at day 4, mouse ESCs adapt to their mechanical environment with different morphology on soft and rigid substrates.
Figure 2-2  Morphology of ESC colonies on substrates of varying elasticity. ESCs on 1 kPa (soft substrate), 10 kPa (intermediate substrate) and glass (rigid substrate) formed round and compact colonies on day 4 when cultured with the addition of LIF in the culture media (+ LIF). ESCs on 1 kPa and 10 kPa substrates still formed round and compact colonies at day 4 even in the absence of LIF from the culture. On the rigid glass substrate, 4 days of culture without LIF resulted in irregularly shaped and widespread colonies (- LIF). Three independent experiments showed similar results. Scale bar, 100 µm.
2.3.2 Effects of substrate elasticity on ESC pluripotency maintenance

The above results showed that ESC morphology is different on soft and rigid substrates after 4 days in culture in the absence of LIF. To find out if these changes also affect ESC pluripotency and the expression of pluripotent transcription factors, I investigated the activity of the pluripotency marker, alkaline phosphatase (AP), in colonies cultured on substrates of different degrees of elasticity (Figure 2-3). After 4 days of the LIF+ culture, the compact and round colonies on 1 kPa and 10 kPa exhibited high AP activity. The numbers of AP-positive ESCs on these substrates were 96% and 95%, respectively. The AP reactivity of the colonies on glass substrate was 98% of all colonies, although the colonies were not as compact as those cultured on the softer substrates. In the cultures without LIF for 4 days, ESCs on the rigid glass substrate exhibited signs of differentiation with little to undetectable AP activity. The AP activity significantly decreased from 98% to 3% on the rigid glass substrate. Strikingly, the percentage of AP-positive ESCs on 1 kPa was maintained at 98%, similar to those in LIF+ conditions. Similar results were obtained from ESCs cultured on the 10 kPa substrate. These results show that soft substrates are able to promote mouse ESC pluripotency even in the absence of LIF at day 4. Next, to examine gene expression patterns of core transcription factors, mouse ESCs deposited on 1 kPa, 10 kPa and glass substrates were cultured in the presence of LIF for 4 days and analyzed by immunofluorescence using Oct4, Nanog and Sox2 antibodies. The colonies on the soft and rigid substrates were then observed using confocal laser scanning microscopes. Figure 2-4 shows the concurrent expression of Oct4, Nanog and Sox2 as revealed by immunofluorescence. The fluorescent signal of Oct4 is shown in green, whereas Nanog and Sox2 are shown in blue and red, respectively (Figure 2-4A). In the presence of LIF, the expression levels of Oct4, Nanog and Sox2
were observed to be homogeneous. However, in the absence of LIF, the stainings of the colonies cultured on 10 kPa and glass substrates were observed to be heterogeneous. In comparison, mouse ESCs cultured on 1 kPa substrate in the absence of LIF exhibited homogeneous colony staining. Figure 2-4B shows the mean fluorescence intensity of Oct4, Nanog and Sox2 calculated from at least 50 cells obtained from random ESC colonies cultured on 1 kPa, 10 kPa and glass substrates with or without LIF at day 4. As expected, the fluorescence intensities of all three of the core transcription factors were high for ESCs cultured on 1 kPa, 10 kPa and glass substrates in the presence of LIF. However, when LIF was removed, and cells cultured without it for 4 days, the cells on 1 kPa substrate had increased expression of Oct4, Nanog and Sox2 compared with the cells cultured on 10 kPa and glass substrates.

To corroborate these findings and to evaluate expression levels of Oct4, Nanog and Sox2 on mRNA level in cultures of ESCs on soft substrates, I analyzed the respective genes by quantitative real-time PCR technique. The expression levels of Oct4, Nanog and Sox2 mRNA in cells cultured on 1 kPa, 10 kPa and glass substrates in the absence of LIF at 4 days are shown in Figure 2-5A. The expression levels of the genes determined by quantitative real-time PCR in soft substrates is shown relative to the levels in cells cultured on glass in the absence of LIF for 4 days. The levels of Oct4, Nanog and Sox2 in cells from 1 kPa substrates were significantly higher than in the cells cultured on glass (p < 0.05). The expression levels of Oct4, Nanog and Sox2 in the cells cultured on 10 kPa substrates were also significantly higher than in the cells cultured on glass (p < 0.05).

Furthermore, mRNA expression levels of additional stem cell regulators of ESCs were also analyzed. Figure 2-5B shows the mRNA expression levels of Stat3, Fgf4, Klf4, Eras and Lin28 (Hadjimichael et al., 2015) in cells cultured on 1 kPa (soft substrate) relative to the cells cultured
on glass in the absence of LIF for 4 days. The gene expression levels of Stat3, Fgf4 and Klf4 in cells cultured on soft 1 kPa substrates were relatively higher than the expression levels of above-mentioned genes in the cells cultured on rigid substrate. However, the gene expression levels of Eras and Lin28 were relatively lower than in the cells cultured on glass ($p > 0.05$). Nonetheless, soft substrates promoted higher expression of Oct4, Nanog and Sox2 in mouse ESCs even in the absence of LIF.
Figure 2-3  Alkaline phosphatase (AP) activities of ESC colonies on varying substrate elasticity. Compact and round colonies of ESCs on soft substrate (1 kPa), intermediate substrate (10 kPa) and rigid glass substrate (50 GPa) stained positive for AP staining at day 4 of culture in the presence of LIF (+LIF). In the absence of LIF for 4 days, ESC colonies on soft and intermediate substrates still stained positive for AP staining showing signs of pluripotency. ESCs on the rigid substrate exhibited signs of differentiation with undetectable AP activity in the absence of LIF at day 4 (-LIF). Three independent experiments showed similar results. Scale bar, 100 µm.
Figure 2-4 Expression levels of Oct4, Nanog and Sox2 in ESC colony. (A) Immunostaining images of mouse ESC colonies expressing Oct4 (green), Sox2 (red) and Nanog (blue). Scale bar, 100 μm. (B) Quantification of mean fluorescence intensity of Oct4, Nanog and Sox2 calculated from at least 50 cells of random colonies. Asterisks indicate statistically significant differences with cells on glass with/without LIF (*p < 0.05, **p < 0.01). Error bars, SD (N = 50); at least three independent experiments.
Figure 2-5 Gene expression levels of stem cell regulators of ESCs. Expression levels of (A) Oct4, Nanog and Sox2 mRNA of ESCs cultured on soft substrate (1 kPa), intermediate substrate (10 kPa) and rigid substrate (50 GPa) at day 4 of culture in the absence of LIF relative to GAPDH mRNA levels. The expression level of the genes was determined by quantitative real-time PCR expressed relative to the levels in cells cultured on rigid substrate in the absence of LIF at day 4. Expressions levels of Oct4 and Nanog on soft and intermediate substrates were higher compared to the levels of cells in rigid substrate. (B) Stat3, Fgf4, Klf4, Eras and Lin28 mRNA of ESCs cultured on soft
substrate (1 kPa) and rigid substrate (glass) without LIF at day 4 relative to GAPDH mRNA levels. The expression level of the genes was determined by quantitative real-time PCR normalized to the levels in cells cultured on rigid substrate in the absence of LIF at day 4. Expressions levels of Stat3, Fgf4 and Klf4 on soft substrate were higher than the levels on rigid substrate. Asterisks indicate statistically significant differences with cells on glass without LIF (*p < 0.05, **p < 0.01). Error bars, SD (N = 4); at least four independent experiments.
2.3.3 The effects of substrate elasticity on cytoskeletal proteins

Previous work in our lab has established that Rho-kinase (ROCK) is downregulated when mouse embryonic fibroblasts (MEF) are induced to become pluripotent on soft substrates (Higuchi et al., 2014). A major downstream factor of RhoA, ROCK, modulates actin cytoskeleton organization and stress fiber formation. The major role of ROCK1 is inactivation of myosin light chain phosphatase which results in actomyosin contraction. ROCK2 through LIM kinases (LIMK) activation causes cofilin phosphorylation that leads to inhibition of actin depolymerization (Figure 2-6A). This pathway is strongly related to stress fiber formation and cell adhesion (Hartmann et al., 2015; Shi et al., 2013). To investigate if mouse ESCs exhibit similar regulation of ROCK, next, I assessed the state of ROCK1 and ROCK2 in early mouse ESCs. I analyzed these genes’ expression levels in early differentiating cells cultured on 1 kPa, 10 kPa and glass substrates in culture medium without LIF at day 4. Figure 2-6B shows the expression levels of RhoA, ROCK1 and ROCK2 signaling genes of mouse ESCs on 1 kPa, 10 kPa and glass substrates in the absence of LIF for 4 days. In this experiment, gene expression levels were determined in cells cultured on soft substrates by qRT-PCR and shown relative to mouse ESCs cultured on rigid glass substrates in the absence of LIF at day 4. The expression levels of RhoA, ROCK1 and ROCK2 did not change significantly ($p > 0.05$). However, when mouse ESCs were cultured on soft substrates, the expression level of ROCK2 was lower compared to the cells cultured on 10 kPa and glass substrates.

To further corroborate the above findings and to reveal the influence of ROCK2 on F-actin organization in mouse ESCs cultured on soft substrates, I examined the appearance of F-actin filaments at the middle and basal layers of ESCs cultured on 1 kPa, 10 kPa and rigid glass
substrates. Rhodamine phalloidin was used to stain F-actin filaments and DRAQ5 was used to visualize cell nuclei. Using laser scanning confocal microscope, middle layer images of the cultured ESCs were obtained and compared. **Figure 2-7A** shows the fluorescent micrographs of mouse ESCs stained for F-actin, and nuclei, obtained from the mid-section of the cells. On the soft substrates both with and without LIF, the cortical F-actin expression was visibly lower than the expression of cortical F-actin of ESCs cultured on glass substrates. Also, cortical F-actin in cells cultured on glass substrates without LIF appeared stiff compared to cortical F-actin of the cells cultured on glass substrates with LIF. Interestingly, the cell nuclei in cells cultured on soft substrates, particularly with the addition of LIF, appeared wrinkled compared to the smooth and larger nuclei of ESCs cultured on the rigid dishes. Next, I evaluated the expression of F-actin at the basal layer of the ESC colonies as shown in **Figure 2-7B**. At the basal layer, cells on soft substrates showed diffused fibers, in both LIF+ and LIF- conditions. The network of these actin filaments on the soft substrates also appeared flexible. On the other hand, cells cultured on glass substrates in the presence of LIF showed high F-actin accumulation at the basal layer with well-defined spot-like appearance, denoting thick actin bundles and adhesion to the cell-substrate. Conversely, in the absence of LIF, well-defined rigid stress fibers were observed away from the cell periphery on the glass substrate. Actin bundles were also observed towards the periphery of the cells, denoting lamellipodia, and at cellular edges of the spread cells, representing filopodia. The appearances of lamellipodia and filopodia-like structures are signs of ESC differentiation. The differences in F-actin appearance and position on the soft and rigid substrates indicate that the actin in these cells has undergone distinct rearrangements. The quantification of mean fluorescence intensity of F-actin at the basal layer of the cells on 1 kPa and glass substrates for
both +LIF and –LIF conditions was calculated as shown in Figure 2-7C. To compare the fluorescence intensities between substrates, fluorescence intensity of DRAQ5 stain was measured in 50 cells from each culture condition; it showed stable expression in all culture conditions. Next, the fluorescence intensity of F-actin in these 50 cells was normalized to the DRAQ5 stain. As expected, the intensity of F-actin staining in cells grown on 1 kPa substrate was significantly lower than in cells grown on rigid glass substrate. It is important to note that the addition of LIF in the culture media on similar substrates, did not alter the fluorescence intensity of F-actin in cells cultured on soft and rigid substrates, respectively. This further illustrates the differences in F-actin on soft and rigid substrates irrespective of the addition of LIF in cell culture.

Previous studies reported integrin as an important surface transmembrane receptor, which senses the substrate, links to the ECM and assembles focal adhesions and other cytoskeletal organizing factors that transmit mechanical force and biochemical signals across the plasma membrane (Talwar et al., 2014; Hou et al., 2013; Tojkander et al., 2012). Adapter protein, Talin is a major component of focal adhesion together with F-actin. Its accumulation initiates focal adhesion complex formation and this formation is integrin-dependent (Shih et al., 2011; Ross et al., 2013). From Figure 2-7, F-actin expression was found to be much lower on soft substrate than on rigid substrates. Since F-actin connects to Talin in the focal adhesion complex, to investigate if focal adhesion complex assembly recruited by integrin is also affected by soft substrates, ESCs were first immunostained specifically for Talin and F-actin. Next, since F-actin is also connected to cell-junction proteins, as a control, ESCs were also immunostained specifically for α-catenin, a linking protein at cell junctions between E-cadherin and F-actin. Figure 2-8 shows the immunostaining images of ESCs on 1 kPa and glass substrates to visualize Talin and actin filaments.
at the basal part of the colonies in the presence and absence of LIF for 4 days. As displayed in Figure 2-8A, the concentration of Talin at the base of the cells is reduced on soft substrates compared to the cells on glass substrates in both LIF+ and LIF- conditions. Strikingly, actin filaments on 1 kPa substrates were greatly disrupted irrespective of the presence of LIF. On the other hand, Talin was concentrated toward the periphery of the colonies cultured without LIF on glass substrates (Figure 2-8A). ESCs cultured on 1kPa substrates in both LIF+ and LIF- conditions have noticeably lower levels of actin filament and Talin compared to cells cultured on glass substrates as quantified in Figure 2-8B. To check the co-localization of both Talin and F-actin, scatter plots based on the bright pixels of Talin that coincide with the bright pixels of F-actin were schemed. As shown in Figure 2-8C, co-localization of F-actin and Talin varies between soft and rigid substrates. However, the co-localization of F-actin and Talin does not differ much between soft substrate with or without LIF. Similar results were observed in cells on glass substrates.

The expression patterns of α-catenin were also distinct in ESCs cultured on 1 kPa and glass substrates (Figure 2-9A). On 1 kPa substrates, the amount of visible α-catenin was lower. Strikingly, actin filaments on 1 kPa substrates were greatly disrupted irrespective of the presence of LIF (Figure 2-9A). In the presence of LIF, ESCs on glass substrates showed higher levels of α-catenin and F-actin as quantified in Figure 2-9B. To check the co-localization of both α-catenin and F-actin, scatter plots based on the bright pixels of α-catenin that coincide with the bright pixels of F-actin were schemed. As shown in Figure 2-9C, both localization of α-catenin and F-actin were increased in cells cultured on the glass substrates by the removal of LIF. However, scatter plots of α-catenin and F-actin in cells cultured on the soft substrates, only F-actin was increased in the absence of LIF.
Figure 2-6 Regulation and expression of RhoA and ROCK in ESCs (A) Schematic diagram of the roles of ROCK1 and ROCK2 downstream of RhoA a cytoskeleton regulatory factor. (B) Gene expression levels of ROCK1, ROCK2 and RhoA mRNA of ESCs cultured on soft substrate (1 kPa), intermediate substrate (10 kPa) and rigid substrate (50 GPa) at day 4 of culture in the absence of LIF relative to GAPDH mRNA levels. The expression level of the genes was determined by quantitative real-time PCR expressed relative to the levels in cells cultured on rigid substrate in
the absence of LIF at day 4. The expression level of ROCK2 was lower compared to the cells cultured on intermediate and rigid substrate. Error bars, SD (N = 3); at least three independent experiments.
Figure 2-7 Actin filaments arrangements in mouse ESCs (A) Fluorescent micrographs of ESCs captured at the mid-layer of mouse ESC colonies cultured on 1 kPa and glass with or without LIF for 4 days stained for rhodamine phalloidin to visualize F-actin (green) and DRAQ5 to visualize cell nuclei. (B) Fluorescent micrographs of ESCs captured at the basal layer of mouse ESC colonies cultured on 1 kPa and glass with or without LIF for 4 days stained to visualize F-actin (green). (C) Quantification of mean fluorescence intensity of F-actin and DRAQ5 calculated from randomly selected 50 cells imaged at the base of the ESC colonies. Asterisks indicate statistically significant differences with cells on glass with or without LIF (*p < 0.05, **p < 0.01). Error bars, SD (N=50). Scale bars, 50 µm.
Figure 2-8 Changes of focal adhesion complex and F-actin assembly in ESCs cultured on varying substrate elasticity. (A) Fluorescent images of ESCs stained for rhodamine phallloidin to visualize F-actin (red) and immune-stained with Talin antibody (green) to represent focal adhesion complex. The cells were cultured on 1 kPa and glass in the presence and absence of LIF for 4 days. The assembly of Talin and F-actin is reduced in soft substrates compared to rigid substrates on both +LIF and -LIF at day 4. (B) Quantification of mean fluorescence intensity of Talin and F-actin in ESCs cultured on soft and rigid substrates on LIF+ and LIF- at day 4. The fluorescence intensities of both Talin and F-actin were lower on cells cultured on soft substrates compared to the cells cultured on rigid substrate. (C) Co-localization scatter plot of Talin intensity to F-actin intensity on LIF+ and LIF- conditions at day 4. Asterisks indicate statistically significant differences with cells on glass with or without LIF (*p < 0.05, **p < 0.01). Error bars, SD (N = 50). Scale bar, 50 µm.
A

1 kPa

Glass

LIF+

LIF−

Alpha-catenin/ F-actin

50 μm

B

Intensity (a.u.)

180
160
140
120
100
80
60
40
20
0

LIF+ LIF− LIF+ LIF−

1 kPa Glass

F-actin Alpha-catenin

C

Alpha-catenin intensity

10^2
10^1
10^0

F-actin intensity

10^2
10^1
10^0

soft/rigid

LIF+

LIF−
Figure 2-9 Changes of cell-junction protein and F-actin assembly in ESCs cultured on varying substrate elasticity (A) Fluorescent images of ESCs stained for rhodamine phalloidin to visualize F-actin (red) and immune-stained with anti-alpha catenin antibody (green). The cells were cultured on 1 kPa and glass in the presence and absence of LIF for 4 days. The staining of alpha catenin and F-actin is reduced in soft substrates compared to rigid substrates on both +LIF and -LIF at day 4. (B) Quantification of mean fluorescence intensity of alpha catenin and F-actin in ESCs cultured on soft and rigid substrates on LIF+ and LIF- at day 4. The fluorescence intensities of both alpha catenin and F-actin were lower on cells cultured on soft substrates compared to the cells cultured on rigid substrate. (C) Co-localization scatter plot of alpha catenin intensity to F-actin intensity on LIF+ and LIF- conditions at day 4. Asterisks indicate statistically significant differences with cells on glass with or without LIF (* p < 0.05, ** p < 0.01). Error bars, SD (N = 50). Scale bar, 50 µm.
2.4 Discussion

ESCs are usually maintained in cell cultures in the presence of LIF to inhibit differentiation (Smith et al., 1988; Williams et al., 1988). However, LIF is often insufficient to inhibit the spontaneous differentiation of ESCs. Recently, several studies on the effects of mechanical stimulation on the function and organization of cells have emerged (Wang et al., 2009, Yip et al., 2013, Yim et al., 2012). Nonetheless, these works have not shown that mechanical signals arising from sensing substrate elasticity can alter expression of pluripotent transcription factors and affect actin-related cytoskeletal network. In this chapter, I showed that we can successfully inhibit the spontaneous differentiation of mouse ESCs in the absence of LIF by culturing the cells on soft substrates. In the absence of LIF, ESCs formed compact round colonies when cultured on soft 1 kPa substrate, in contrast to the spread colonies observed on the glass substrate (Figure 2-2). Further, ESCs on soft substrates without LIF showed persistent signs of pluripotency with positive AP activity (Figure 2-3). This result is consistent with previous reports showing that cell morphology regulates the commitment of differentiation specification (McBeath et al., 2004, Engler et al., 2006; Gupta et al., 2015; Mammoto et al., 2012; Sun et al., 2012). Oct4, Nanog and Sox2 expression levels were also higher in cells cultured on soft substrates in the absence of LIF (Figure 2-4 and Figure 2-5). Evaluation with stem cell regulators of embryonic stem cells revealed that ESCs on soft substrates without LIF have higher expression levels of Stat3 and Klf4 which could promote the expression of Oct4, Nanog and Sox2. The expression of Fgf4 which is essential for the survival of post-implantation mouse embryo is also higher in cells on soft substrates supporting the premise that cells grown on soft substrates are more pluripotent compared to cells on rigid substrates (Figure 2-5). However, the expression levels of Eras and Lin28 were
slightly lower in cells grown on soft substrates comparing to the cells grown on rigid substrate (Figure 2-5). This could be due to the fluctuations of gene expressions in ESCs in the absence of LIF. Since stem cell culture often contains spontaneously differentiated cells (Smith et al., 2017), the lower expression of Eras and lin28 could be due to these fluctuations (Figure 2-5B).

The assembly of actin cytoskeleton is mediated by RHO-family GTPases. RhoA is involved in the organization of stress fibers and focal adhesions (Macbeath et al., 2004; Ohgushi et al., 2010). RhoA activates ROCK, which regulates actin dynamics (Taylor et al., 2011). In this chapter, ROCK2 showed reduced expression when cells were cultured on 1 kPa substrates. Since ROCK inhibits de-polymerization of actin filaments (Bhadriraju et al., 2007), this result suggests that actin polymerization is down-regulated on the soft substrates. In addition, the dissimilar appearances of F-actin filaments at the cell middle and basal layers on the soft and rigid substrates in Figure 2-7 suggest different levels of F-actin assembly in cells on these substrates (irrespective to the addition of LIF) corresponding to the different levels of external mechanical forces as reported previously using other cell types (Orlova and Egelman, 1993). Reduction of α-catenin expression on soft substrates in LIF-condition could be a response to E-cadherin inhibition during differentiation (illustrated in Figure 2-10). Here, the immunostaining experiments revealed that the intensities of α-catenin, Talin and F-actin were strongly influenced by the substrate elasticity (Figure 2-8 and Figure 2-9).

Cells attach to the ECM through integrin, and at the cytoplasmic site, integrin is attached to Talin that assembles focal adhesion complex. Integrin works as a signaling platform at the cell membrane that informs the cell about ECM mechanical environment and provides an anchoring point for F-actin which readily responds to mechanical challenges by re-inforcing its structure.
through formation of new stress fibers. Force generation in stress fibers is regulated by RhoA which increases cell tension through ROCK. Here, I have shown that mechanical challenges provided by rigid substrate increase the number of focal adhesions (represented by Talin) and enriches associated cytoskeletal structure to adapt to mechanical challenges through ROCK (Figure 2-10).

Thus, the reduction of mechanical stimuli in ESCs grown on soft substrates repressed Talin and consequently reduced actin filament accumulation with the downregulation of ROCK2. Considering these data, I hypothesized that F-actin filaments may be generally disrupted in pluripotent stem cells and are activated in response to mechanical stress, which eventually results in the loss of pluripotency.
Figure 2-10 Schematic of possible signaling crosstalk presented in this chapter. Mechanical signals delivered by rigid substrate increase the number of focal adhesions complex (Talin) and enriches associated F-actin structure to adapt to mechanical challenges through ROCK. ONM (Outer nuclear membrane) and INM (Inner nuclear membrane)
Chapter 3 Control of embryonic stem cell pluripotency by nucleo-cytoskeletal network disruption

3.1 Introduction

The high plasticity of ESC pluripotency state often causes heterogeneous expressions and differentiation of the cells (Mummery et al., 1990; Nishikawa et al., 2007). This work so far has shown that both biochemical and mechanical signals arising from sensing the substrate elasticity can regulate the transcriptional factors of mouse ESCs. In the previous chapter I have shown that actin filaments which were inactive in pluripotent cells became activated in response to mechanical stress. The focal adhesions became more pronounced as shown by Talin expression on rigid substrates, resulting in the loss of pluripotency. Considering this finding, I hypothesized that pluripotency can be regulated by the mechanical signals transmitted from the substrate into the nucleus via F-actin filaments. Previous studies have reported the possible roles of the cytoskeleton in transmitting mechanical signals to the nucleus which were able to alter the nuclear shape and intracellular matrix (Webster et al., 2009; Alam et al., 2014; Li et al., 2011). However, the molecular mechanisms of mechanical signal transmission to the ESC nucleus and the subsequent regulation of the pluripotency genes are unknown.

The physical connection between the nucleus and the cytoskeleton is crucial for various cellular functions such as cell migration and division. Therefore, a strong and stable connection is important to facilitate such functions (Lombardi et al., 2011; Isermann and Lammerding, 2013). The nucleus stores the genetic materials of the cells. The nuclear membrane is comprised of the inner and outer membranes that separate the nucleoplasm from the cytoplasm. The inner
membrane interacts with nuclear lamina, which consists of lamin, emerin and the SUN proteins (Crisp and Burke, 2008; Wagner and Krohne, 2007). Nuclear lamina also connects with chromatin and regulates its organization and gene expression (Alam et al., 2014). KASH (Klarsicht, ANC-1 and SYNE1 Homology) domain proteins are located at the outer nuclear membrane. Four genes of nesprin family, which produce KASH domain proteins, have been identified and they are made up of many splicing variants. Of all these, Nesprin 1 and Nesprin 2 giant isoforms are the largest proteins (1 MDa and 800kDa in size). These multi-domain proteins contain a paired calponin-homology (CH) domain at the N-terminus, which binds and enables the interaction with actin filaments. The central domain contains spectrin repeats, whereas the C-terminus contains the KASH domain that interacts with the SUN proteins at the inner nuclear membrane. As shown in Figure 3-1, the Nesprin and SUN proteins form a connection between the nuclear and the cytoskeletal components, and are called the linker of nucleoskeleton and cytoskeleton (LINC) complexes (Zhang et al., 2009; Zhang et al., 2004; Luke et al., 2008; King et al., 2014).

As a follow-up to the results in the previous chapter that showed F-actin-mediated regulation of pluripotency, here I investigate if the direct disruption of the F-actin connection between the nucleus and the cell membrane can alter the expression of the pluripotency genes. Since Nesprin 1 and Nesprin 2 bind with cytoplasmic actin filaments, the disruption of their connection may change actin organization and inhibit the signal transmission from rigid substrates to the nucleus, resulting in the maintenance of ESC pluripotency even in the absence of LIF.
Figure 3-1 Schematic diagram of physical connection of F-actin from substrate to cell nucleus. LINC complex is formed by the KASH domain proteins (Nesprin 1 and Nesprin 2) at the outer nuclear membrane (ONM) and SUN domain proteins located at the inner nuclear membrane (INM) which also connects with nuclear lamina. Nesprin 1 and Nesprin 2 bind to actin filaments
3.2 Materials and methods

3.2.1 Cell culture

Cell line E14Tg2a of mouse embryonic stem cells (ESCs) was used in this study and they were routinely cultured on plastic culture dishes (Falcon; BD Biosciences, Franklin Lakes, NJ; catalog numbers 353003, 353001, 353046 and 353004) coated with 0.1% gelatin (Sigma-Aldrich, MO, USA). The cells were incubated at 37°C in an atmosphere of 5% CO₂ supplemented with Dulbecco’s Modified Eagle’s Medium (DMEM, D6046; Sigma-Aldrich, MO, USA) containing 10% fetal bovine serum (FBS; ThermoFisher Scientific, MA, USA) 1% penicillin antibiotics (Sigma-Aldrich, MO, USA), 1% streptomycin (Sigma-Aldrich, MO, USA), 1% Gibco GlutaMAX-I L-glutamine (ThermoFisher Scientific, MA, USA), 1% minimum essential medium (MEM) non-essential amino acids (ThermoFisher Scientific, MA, USA), 1% nucleosides (Sigma-Aldrich, MO, USA), 1% sodium pyruvate (Sigma-Aldrich, MO, USA), 0.1% 2-mercaptoethanol (Sigma-Aldrich, MO, USA) and 0.1% leukemia inhibitory factor (LIF; NACALAI TESQUE, INC., Japan). Media were changed every two days and cells were harvested when they are 70% confluent. The cells were cultured up to 4 days in culture media containing LIF to maintain the undifferentiated state of the mouse ESCs. To observe the spontaneous differentiation of the cells, ESCs were cultured in culture media with the absence of LIF for 4 days (unless stated otherwise).

3.2.2 Dominant negative experiment of Nesprin KASH DNA constructs and transfection

To generate dominant negative KASH (also known as KLS) domain of Nesprin 1 and Nesprin 2 with mEGFP, the KASH domains from mouse Nesprin 1 giant (NM_001347711.1) and Nesprin 2 giant (NM_001005510.2) were amplified by PCR (primer sequences in Table 3-1) and inserted in
The mEGFP-pcDNA3mod plasmid (provided by Dr. Machiyama Hiroaki). Nesprin 2 KASH domain was inserted at the N-terminal of GFP domain at the XbaI/ClaI restriction sites. Nesprin 1 KASH domain was inserted at the NotI and ApaI restriction sites. To co-express both the Nesprin 1 KASH domain and Nesprin 2 KASH domain genes, T2A peptides were inserted ClaI/NotI restriction sites. To create stable cell lines overexpressing KASH domains of Nesprin 1 and Nesprin 2, mEGFP-Nesprin 2 KASH - T2A - Nesprin 1 KASH was subcloned from mEGFP-Nesprin 2 KASH-T2A-Nesprin 1 KASH-pcDNA3mod into pT2b-EF-MCS3-pP transposon vector (provided by Dr. Takai Akira) to create pT2b-EF-MCs3- mEGFP-Nesprin 2 KASH - T2A - Nesprin 1 KASH -pP. To compare the roles of Nesprin 1 KASH and Nesprin 2 KASH in mouse ESCs, mEGFP-Nesprin 2 KASH was subcloned from mEGFP-Nesprin 2 KASH-T2A-Nesprin 1 KASH-pcDNA3mod into pT2b-EF-MCS3-pP transposon vector to create pT2b-EF-MCs3-mEGFP-Nesprin2 KASH-pP. Finally, as a mock control with mEGFP alone, mEGFP was subcloned into the provided tol2b vector to create pT2b-EF-MCs3-mEGFP-pP vector. To clone the nesprin constructs into the Tol2b vector, the KASH domain genes were amplified from the mEGFP-Nesprin 2 KASH-T2A-Nesprin 1 KASH-pcDNA3mod with primers containing EcoRI and Kozak sequence and then ligated into the EcoRI-SpeI sites in pT2b vector; Forward 5’ – AAAGAATTCCACCATGGTGAGCAAGGGCG – 3’ and reverse primers of mEGFP, mEGFP-Nesprin 2 KASH and mEGFP-Nesprin 2 KASH-T2A-Nesprin 1 KASH are 5’ – TTTACTAGTTTACTTGTACAGCTCGTCCATGCGG – 3’, 5’ – TTTACTAGTTTACTGTTGGAGGAGGTGGCCCG – 3’ and 5’ – TTTACTAGTTTACTGTTGGGTATATCTGAGCATCGGATG – 3’ respectively and verified by gene sequencing. These transposon constructs were transfected into mouse ESCs together with helper transposase using Lipofectamine 2000 (ThermoFisher Scientific, MA, USA) following...
the manufacturer’s instructions. The cells were left to proliferate for 3 days and harvested with puromycin selection and confirmed by FACS at day 7.

3.2.3 Quantification of gene expression

Gene expression levels of the undifferentiated ESCs cultured in the presence of LIF as well as the differentiated ESCs cultured in the absence of LIF for 4 days on 1 kPa, 10 kPa and glass substrates were quantified by quantitative real-time polymerase chain reaction (qRT-PCR). Firstly, RNA of the differentiated and the undifferentiated ESCs was isolated using RNeasy mini kit (Qiagen, Germany) and converted into cDNA using Omniscript RT Kit (Qiagen, Germany) according to manufacturer’s instructions. The cDNA was analyzed using primers conforming to *Mus Musculus* sequences as listed in Table 3-1 for qRT-PCR. To perform the analyses, a mixture of Thunderbird SYBR qPCR mix (TOYOBO, Japan), gene-specific primers (0.4 µM), cDNA template (300 ng) and distilled water was prepared, making a total of 8 µL for each well. The mixture was reacted in a CFX96 real-time PCR analysis system (Bio-Rad, Hercules, CA, USA) with each sample performed in triplicate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. The qRT-PCR conditions used for the experiments were: 98°C for 30 s, 33 cycles of 98°C for 5 s, 54.5°C (Nanog), 57.3°C (Oct4), 54.6°C (Sox2), 53.4°C (GAPDH), 55°C (Lin28, Rex1, Eras, Klf4, Fgf4, Stat3, RhoA, ROCK1 and ROCK2) for 30 s respectively, 50°C for 5 s and 95°C for 5 s. Expression of Oct4, Nanog, Sox2, Lin28, Rex1, Eras, Klf4, Fgf4, Stat3, RhoA, ROCK1 and ROCK2 proteins at the mRNA level was normalized to GAPDH, which showed very little variation between the undifferentiated and differentiated ESCs.
**Table 3-1 Primers used for quantitative real time RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5’-GCACAGTCAAGGCCGAGAAT-3’</td>
<td>5’-GCCTTCTCCATGGGTGGA-3’</td>
</tr>
<tr>
<td>Oct4</td>
<td>5’-TCTTTACCACAGGCCCCCGGCTC-3’</td>
<td>5’-TGCGGGCAGCATGAGGAGATCC-3’</td>
</tr>
<tr>
<td>Nanog</td>
<td>5’-AGGGTGCTGCTACTGAGATGCTCTG-3’</td>
<td>5’-CAACCCTGGTTTTCTGCCCAGG-3’</td>
</tr>
<tr>
<td>Sox2</td>
<td>5’-TAGAGCTAGACTCGCCGCTGGA-3’</td>
<td>5’-TTGCCCTAAACAAGACCACCGAAA-3’</td>
</tr>
<tr>
<td>Lin28</td>
<td>5’-CCAATGTAATCTCTCACCTTCTG-3’</td>
<td>5’-GCTTTGTTTCTAGCAGTAAACA-3’</td>
</tr>
<tr>
<td>Rex1</td>
<td>5’-ATTCCATGATATGTTCAACAGG-3’</td>
<td>5’-TGTTGACTACTGCCAAGTTGGCC-3’</td>
</tr>
<tr>
<td>Eras</td>
<td>5’-TGTGGCTGCTCTGATGCTTAA-3’</td>
<td>5’-TCATAAAACACAGTCAATGAGG-3’</td>
</tr>
<tr>
<td>Klf4</td>
<td>5’-AGTTCTCACTCAGTCCTGACT-3’</td>
<td>5’-AACACATTTTCTGCTTAA-3’</td>
</tr>
<tr>
<td>Fgf4</td>
<td>5’-AAGACGGTCTCAGGCCCAGGCT-3’</td>
<td>5’-AGCAGTAGCCGCGCTTGCT-3’</td>
</tr>
<tr>
<td>Stat3</td>
<td>5’-CTTGCTCACCTCACAGAGCACC-3’</td>
<td>5’-GATCCATGTCAAAGTGAGGCG-3’</td>
</tr>
<tr>
<td>Nesprin1</td>
<td>5’-CGTATCATGCTGCTGCTAACA-3’</td>
<td>5’-TTGACTAATTTAATCGGTGATCTTCT-3’</td>
</tr>
<tr>
<td>Rock1</td>
<td>5’-TTCATGTCGCCAGCTGCTAACC-3’</td>
<td>5’-TTGACAGCAGCTTGAGGAG-3’</td>
</tr>
<tr>
<td>Rock2</td>
<td>5’-TTCACGTCCCACCTGTTACC-3’</td>
<td>5’-GTGGCACCCTACGCGACTCTA-3’</td>
</tr>
</tbody>
</table>
3.2.4 Immunofluorescence

The sources of primary antibodies used in the experiments were Oct4 mouse monoclonal antibody (sc-5279, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Sox2 rabbit polyclonal antibody (ab97959, Abcam, UK), Nanog goat polyclonal antibody (sc-30328, Santa Cruz Biotechnology). Generally, ESCs were cultured on culture dishes for 4 days in the presence of LIF in the culture media to sustain the pluripotency of the cells and in the absence of LIF to accommodate spontaneous differentiation of the cells. After 4 days, the cells were washed with 1 × phosphate-buffered saline (PBS; NACALAI TESQUE, INC., Japan) once before fixing with 4% paraformaldehyde (PFA; EMS, Hatfield, PA, USA) for 5 min at room temperature. Fixed cells were then washed with 1 × PBS thrice for 5 min each and incubated in 0.01% TWEEN®20 (Sigma-Aldrich, MO, USA) in PBS for 5 min. The samples were subsequently blocked with blocking agent, CAS-Block (Thermo Fisher Scientific, MA, USA) solution for 30 min at room temperature. Primary antibodies were diluted at 1:100 with CAS-Block blocking agent and incubated either for 1 h at room temperature or overnight at 4°C. After 1 h or the next morning, the cells were washed with 1 × PBS thrice for 5 min each. The fluorescent secondary antibodies used in the experiment were Invitrogen anti-mouse Alexa Fluor 488 IgG (Thermo Fisher Scientific, MA, USA), Invitrogen anti-rabbit Alexa Fluor 594 IgG (Thermo Fisher Scientific, MA, USA) and Invitrogen anti-goat Alexa Fluor 647 IgG (Thermo Fisher Scientific, MA, USA) diluted at 1:400 with CAS-Block blocking agent and the samples were stained for 1 h at room temperature. Finally, the samples were rinsed with 1 × PBS thrice for 5 min each before imaging. Samples were examined with an Olympus FV 1000 (Olympus, Hamilton, Bermuda) laser scanning confocal microscope with 60× 1.4NA oil immersion objective.
3.2.5 Western blot analysis

At day 4 of culture, cells were first washed with PBS twice and homogenized in 10% SDS (sodium dodecyl sulfate) and incubated in room temperature for 1 h and added with 1M Dithiothreitol (DTT) and 4X Laemmli buffer. The protein extracts were resolved with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% Mini-PROTEAN TGX gel (Bio-Rad, Hercules, CA, USA); and transferred into polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) and processed for immunodetection. The protein containing membranes were initially blocked in 5% skim milk in TBS-tween20 and incubated for 1 h in room temperature. The antibodies used in the experiments were Oct4 mouse monoclonal antibody (sc-5279, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Sox2 rabbit polyclonal antibody (ab97959, Abcam, UK), Nanog goat polyclonal antibody (sc-30328, Santa Cruz Biotechnology) and anti-alpha tubulin mouse monoclonal antibody (Sigma-Aldrich, MO, USA) diluted at 1:500 dilutions. Secondary HRP-conjugated anti-rabbit and anti-mouse antibodies diluted at 1:3000 (GE Healthcare Life Sciences, PA, USA) were used later. Immunoblots were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, MA, USA) on a ImageQuant LAS 4000 (GE Healthcare Life Sciences, PA, USA) software. Quantification was performed using ImageJ software (version 1.47) and alpha Tubulin was used as a loading control.

3.2.6 RNA-interference

Mouse Nesprin 1 knockdown was accomplished by plasmid based small interference RNA (siRNA). The siRNA specific for mouse Nesprin 1 (s234287) ambion (Sigma-Aldrich, MO, USA) was
transfected into mouse ESCs using Lipofectamine 2000 as per manufacturer’s instructions. For negative control, scrambled siRNA was also purchased from ambion (Sigma-Aldrich, MO, USA).

3.2.7 Fluorescent reporter constructs

Nanog and Oct3/4 mouse reporters’ constructs were based on a pRedZeo-lenti pluripotency reporter system (System Bioscience). Mouse Sox2 reporter construct was based on a pGreenFire reporter system (System Bioscience). Using PCR amplification, BamH I/Sal restriction sites were initially added to the cDNA of a Venus fragment to make a Nanog-venus reporter construct. Then the fragment was replaced with the RFP of a SR10044-PA (System Biosciences) plasmid. Next, using PCR, cDNAs of an Oct3/4 reporter (SR10043-PA, System Biosciences) and mKate2 containing Cla I/BamH I sites and BamH I/Sal I sites were amplified to make a Oct3/4-mKate2 reporter. Then at the Cla I/BamH I and BamH I/Sal I restriction sites, Oct3/4 reporter and mKate2 fragments were replaced with Nanog reporter and Venus. Mouse Sox2 reporter were made by adding the Cla I/BamH I restriction sites to the Sox2 response element (SR20071-PA, System Biosciences) and amplified by PCR. Afterwards, amplified KusabiraOrange2 (KO2) was replaced with the copGFP sequence of TR010-PA1. These constructs were then transferred into Escherichia coli DH5α. Using Qiagen miniprep kit, plasmid purification was conducted according to the manufacturer’s protocol (Qiagen). Later, to make lentivirus, 293T cells were transfected with the lentivirus plasmid using FuGENE®HD (Promega). Initially, lentivirus coded with a single reporter was transfected to mouse ESC. To segregate the reporter-positive cells, FACS (Aria III, BD Biosciences) was used. The procedure was repeated until all the 3-reporter positive cells were obtained.
3.2.8 Image analysis

Images were saved as original imaging format (.oif) files with dimensions of 2048 × 2048 pixels and a file depth of 16 bits. All image analyses were performed using ImageJ software (version 1.47).

3.2.9 Statistical analyses

Data presented in this work were obtained from at least three independent experiments and presented as means with standard deviations (SD). Student’s t-test was performed to calculate the statistical significance among data sets with values of $p < 0.05$ considered significant.
3.3 Results

3.3.1 Nucleo-cytoskeletal coupling disruption with dominant negative Nesprin 1 and Nesprin 2 constructs

Nesprins are multi-domain proteins that connect nucleoskeleton to the cytoskeleton via the LINC complex. To disrupt F-actin connection through the LINC complex to the nucleus, I targeted Nesprin 1 and Nesprin 2 since they connect F-actin to the nucleus via their CH domain at actin binding sites. In this experiment, I stably transfected dominant negative Nesprin 1 and Nesprin 2 constructs containing the C-terminal KASH domain fused to mEGFP into mouse ESCs. To reveal the roles of Nesprin 1 and Nesprin 2 in ESCs (Figure 3-2), cell lines expressing dominant negative KASH domain of Nesprin 2 fused with GFP (GFP N2KASH), dominant negative KASH domains of both Nesprin 1 and Nesprin 2 fused with GFP (GFP N2KASH/N1 KASH) and a mock control with GFP (GFP) was generated (Figure 3-2A).

Each of the three cell lines was cultured in the absence of LIF for 4 days and on the fourth day, the activity of the pluripotency indicator alkaline phosphatase (AP) in the cells using alkaline phosphatase (AP) assay was examined (Figure 3-2B). The colonies overexpressing dominant negative KASH domains of Nesprin 1 and Nesprin 2 showed higher populations of colonies with positive AP activity compared to the colonies with dominant negative KASH domain of Nesprin 2. As expected, the control cells with mock GFP did not show any signs of AP activities. Strikingly, the morphologies of the colonies in the control cells were spread compared to the cells in the dominant negative KASH domains of Nesprin 1 and Nesprin 2. These results suggest that ESCs overexpressing KASH domains of Nesprin 1 and 2 are able to maintain the pluripotency in the absence of LIF at day 4. To verify this, I examined the immunofluorescence expressions of Oct4
and Nanog in the cell lines with the mutated nesprins constructs. After 4 days of culture, the cells were immunostained with Oct4 and Nanog and the colonies were observed using confocal laser scanning microscopes. **Figure 3-2C** shows the concurrent immunofluorescence of Oct4 and Nanog. Oct4 expressions are shown in red, whereas those of Nanog are shown in blue. The fluorescent micrographs also show the localization of GFP in control cells and at the nuclear membranes of ESCs with dominant negative KASH domain nesprin constructs. The expressions of Oct4 and Nanog were observed to be homogeneous in the dominant negative of KASH domains of Nesprin 1 and Nesprin 2 (GFP N2 KASH /N1 KASH). Both cell lines with dominant negative KASH domain Nesprin 2 and dominant negative KASH domain Nesprin 1 and Nesprin 2 have increased expressions of Oct4 and Nanog compared with the expressions in the control cells. Next, using real time quantitative PCR (qRT-PCR), the gene expression levels of the transcriptional factors, Oct4, Nanog and Sox2 were measured relative to the levels in mock GFP ESCs on glass in the absence of LIF for 4 days. As depicted in **Figure 3-3**, remarkably, the overexpression of the dominant negative KASH domain of Nesprin 1 and Nesprin 2 (GFP N2KASH/N1KASH) showed significantly higher expression levels ($p < 0.01$) of Oct4, Nanog and Sox2 compared to the ESCs overexpressing dominant negative KASH domain Nesprin 2 and mock GFP. To further evaluate the pluripotency state of the cells, expressions of pluripotency marker genes Rex1, Stat3 and lamin A were also calculated to examine the differentiation state of the mutant cells. The expression of Rex1 on the overexpressed KASH domain of Nesprin 1 and 2 was significantly higher than the mock GFP cells ($p < 0.05$). In addition, the lower expression of lamin A in the dominant negative KASH domain of Nesprin 1 and 2 compared to the control cells shows signs of pluripotency (**Figure 3-3**). The results reveal that the overexpression of dominant
negative KASH domain of both Nesprin 1 and Nesprin 2 has a significant impact on the pluripotency of mouse ESCs compared to that of Nesprin 2 alone.

In the previous chapter, it was suggested that varying substrate elasticities cause different levels of F-actin regulation as a response to the different degrees of external mechanical forces. Since ROCK regulates F-actin dynamics (Taylor et al., 2011) and inhibits the depolymerization of the filaments (Bhadiraju et al., 2007), in this work, the gene expression level of ROCK2 was also measured to further uncover the mechanism of F-actin regulation in dominant negative KASH domain Nesprin 1 and 2 (Figure 3-3). In the previous chapter, ROCK2 showed reduced expression when cultured on 1 kPa, suggesting more actin depolymerizations on the soft substrates. In this experiment, surprisingly, the expression of ROCK2 is upregulated in GFP N2KASH/N1KASH.

Next to examine the capability of the mutant cells to sustain the pluripotency longer than 4 days, I cultured GFP N2KASH/N1KASH, GFP N2KASH and GFP ESCs for 7 days on rigid substrates in the absence of LIF. Similar to the examination as shown in Figure 3-3, using real time quantitative PCR (qRT-PCR), the gene expression levels of stem cell regulators of ESCs Oct4, Nanog, Sox2, Rex1 and lamin A were measured relative to the levels in mock GFP ESCs on glass in the absence of LIF for 7 days. As shown in Figure 3-4, the overexpression of the dominant negative KASH domain of Nesprin 1 and Nesprin 2(GFP N2KASH/N1KASH) still sustained the pluripotency of ESC with higher expression levels of Oct4 and Sox2 compared to the mock GFP. However, the expression of Nanog and Rex1 were not significantly higher compared to the control cells. Lamin A still showed lower expression in dominant negative KASH domain of Nesprin 1 and 2 compared to the mock GFP cells.
To examine the regulation of ROCK, the mRNA expression levels of both ROCK1 and ROCK2 were evaluated \((\text{Figure 3-4})\). Remarkably the expression of ROCK2 was still higher in GFP N2KASH/N1KASH at day 7 in the absence of LIF. However, the expression of ROCK1 was lower in GFP N2KASH/N1KASH compared to control GFP cells, thus indicating different regulation mechanisms of ROCK in ESCs. Further evaluation with Western blot analysis \((\text{Figure 3-5A})\) and quantification \((\text{Figure 3-5B})\) of Oct4, Nanog and Sox2 relative to alpha tubulin as loading control showed that at day 7, GFP N2KASH/N1KASH has significantly higher protein expression levels \((p < 0.05)\) of Oct4 and Nanog compared to the control cells. Together these data suggest that although variability in gene expression was observed, overexpression of dominant negative KASH domain of Nesprin 1 and 2 can still sustain ESC pluripotency in the absence of LIF for 7 days on rigid substrate.
Figure 3-2 Nucleo-cytoskeleton disruption using dominant negative Nesprin 1 and Nesprin 2. (A) Dominant negative KASH Domain Nesprin 1 and Nesprin 2 constructs. (B) AP activity of control cells (GFP), dominant negative KASH Domain Nesprin 2 (GFP-N2KASH) and dominant negative KASH Domain Nesprin 1 and Nesprin 2 (GFP-N2KASH/N1KASH) in the absence of LIF for 4 days. (C) Immunofluorescence images of mouse ESCs of GFP, GFP-N2KASH and GFP-N2KASH/N1KASH in the absence of LIF for 4 days to visualize Oct4 (red) and Nanog (Blue). Scale bars, 100 µm.
Figure 3-3 Gene expression levels of undifferentiated marker gene. Gene expression levels of Oct4, Nanog, Sox2, Rex1, Stat3 and Lamin A and cytoskeleton marker gene ROCK2 mRNA of GFP, GFP-N2KASH and GFP-N2KASH/N1KASH relative to GAPDH mRNA levels in the absence of LIF for 4 days. Error bars, SD (N = 4); at least four independent experiments. Asterisks indicate statistically significant differences with GFP ESCs without LIF (*p < 0.05, ** p < 0.01).
Figure 3-4 Gene Expression levels of undifferentiated marker gene. Expression levels of Oct4, Nanog, Sox2, Rex1 and Lamin A and cytoskeleton marker gene ROCK1 and ROCK2 mRNA of GFP, GFP-N2KASH and GFP-N2KASH/N1KASH relative to GAPDH mRNA levels in the absence of LIF for 7 days. Error bars, SD (N = 4); at least four independent experiments. Asterisks indicate statistically significant differences with GFP ESCs without LIF (*p < 0.05, ** p < 0.01).
Figure 3-5 Western blot (A) analysis and (B) quantification of GFP, GFP-N2KASH and GFP-N2KASH/N1KASH samples in the absence of LIF for 7 days detecting Oct4, Nanog and Sox2 relative to alpha tubulin as loading control. Error bars, SD (N = 3); at least three independent
experiments. Asterisks indicate statistically significant differences with GFP ESCs without LIF at day 7 (*p < 0.05, ** p < 0.01).
3.3.2 Nucleo-cytoskeletal coupling disruption using Nesprin 1 depletion

The overexpression of dominant negative KASH domain of both Nesprin 1 and Nesprin 2 sustained the pluripotency of mouse ESCs significantly compared to the dominant negative of KASH domain of Nesprin 2 alone, suggesting a plausible role of Nesprin 1 in pluripotency regulation. Here I used RNAi to reveal if the depletion of Nesprin 1 in mouse ESCs is sufficient to maintain the pluripotency in the absence of LIF. In this experiment, oligos for siRNA targeting Nesprin 1 was purchased from Ambion, Life Technologies. The oligos were transfected into mouse ESCs to target different exons in the giant isoform of Nesprin 1. After 96 hours post transfection, the Nesprin 1 knockdown cells were evaluated for signs of pluripotency.

Next, using real qRT-PCR, the gene expression levels of the transcriptional factors, Oct4, Nanog, Sox2, Rex1, Lin28, klf4, Stat3 and lamin A were measured relative to control cells with scrambled siRNA cultured on glass bottom dishes in the absence of LIF for 4 days (Figure 3-6). ESCs transfected with siRNA to deplete Nesprin 1 did not show high expressions of the pluripotency markers. Surprisingly, cells transfected with siRNA scramble showed higher mRNA expression levels of Nanog, Sox2, Rex1, Lin28, Klf4 and Stat3 compared to the cells transfected with siRNA Nesprin 1. As shown in Figure 3-6, Oct4 and lamin A expressions were slightly higher in siRNA Nesprin 1 transfected ESCs. To confirm that the changes to Nesprin 1 levels did occur in the experiment, qRT-PCR was used to measure its gene expressions because the Nesprin isoform antibodies are not available. The primers used in the experiment to amplify Nesprin 1 were located at the two actin binding CH domains (Figure 3-2A), which showed that Nesprin 1 was downregulated in mouse ESCs transfected with Nesprin 1 siRNA compared to the control cells without the Nesprin 1 siRNA in Figure 3-6. Together these results show that the depletion of
Nesprin 1 did not support mouse ESC pluripotency maintenance in the absence of LIF at day 4. Establishing efficient siRNA knockdown in mouse ESCs have been reported to be difficult (Chen et al., 2007). Therefore, to further confirm the results from Figure 3-6, fluorescence-activated cell sorting (FACS) with mouse ESCs expressing fluorescent reporters for Oct4, Sox2 and Nanog were conducted. Four culture dishes of mouse ESCs expressing fluorescent reporters of Oct4, Nanog and Sox2 were cultured on: (1) rigid dishes without any drugs as negative control, (2) added with transfection drug FuGENE®HD (Promega, USA) as positive control, (3) added with transfection drug FuGENE®HD and siRNA scramble, and (4) added with transfection drug FuGENE®HD HD and Nesprin 1 siRNA, and left to proliferate for 6 days to examine the pluripotency of the transfected cells. As depicted in Figure 3-7, in the standard culture condition without LIF for 6 days, small populations of the cells cultured without any drugs expressed lower levels of Oct4 and Sox2 reporters, exhibiting low levels of pluripotency. On the other hand, in the remaining three rigid glass dishes that lack LIF but contain: (1) transfection drug only, (2) transfection drug with siRNA scramble, and (3) transfection drug with Nesprin 1 siRNA, they all showed narrow peaks shifted toward the right of the Oct4, Nanog and Sox2 FACS histograms, thus indicating ESC pluripotency. Together these results reveal that the addition of the transfection drug FuGENE®HD affects the expression of the core pluripotency transcription factors even in the absence of LIF for 6 days. Therefore, the data shown in Figure 3-6 and Figure 3-7 are not sufficient to clarify the impact of Nesprin 1 siRNA in sustaining the pluripotency of ESC.
Figure 3-6 Gene Expression levels of pluripotency markers. Expression levels of Oct4, Nanog, Sox2, Rex1, Lin28, Klf4, Stat3, Lamin A and Nesprin 1 mRNA in mouse ESCs with Nesprin 1 siRNA and scrambled siRNA relative to GAPDH mRNA levels. All cells were cultured in the absence of LIF for 4 days. Error bars, SD (N = 4); at least four independent experiments. Asterisks indicate statistically significant differences with cells with scramble siRNA without LIF (*p < 0.05, ** p < 0.01).
**Figure 3-7** FACS histogram of Oct4, Nanog and Sox2 reporters in mouse ESCs. Population of intensity distribution of the core transcription factors Oct4, Nanog and Sox2 reporters in transfected mouse ESCs without LIF for 4 days. Red line indicates population of cells without any transfection drug, green line indicate population of cells transfected with transfection reagent, blue line indicate population of cells transfected with siRNA Nesprin 1 with transfection reagent, and black line indicate population of cells transfected with siRNA scramble with transfection reagent.
3.4 Discussion

In the previous chapter, the experiments showed two important findings; first, soft substrates disrupt the accumulation of F-actin filaments, and second, soft substrates maintain mouse ESC pluripotency by sustaining the expression of the transcription factors. Since F-actin filaments physically connect the nucleus to the cell membrane (Lombardi et al., 2011; Isermann and Lammerding, 2013), it is possible that the disruption of the filaments blocks the connection and transduction of extracellular mechanical signals, which subsequently maintains the expression of the pluripotency transcriptional factors in the ESC nucleus. The results in the chapter support this proposed mechanism of pluripotency maintenance, whereby nucleo-cytoskeleton disruption using dominant negative KASH domain of Nesprin 1 and Nesprin 2 significantly increased pluripotency gene expressions in mouse ESCs in the absence of LIF. Overexpression of dominant negative KASH domain of Nesprin 1 and 2 have high AP activity (Figure 3-2) and expressed higher levels of Oct4, Nanog, and Sox2 (Figure 3-3). Further evaluation with stem cell regulators Rex1, Lin28 and Stat3 also showed high expressions in the dominant negative KASH domain Nesprin 1 and 2 in the absence of LIF at day 4 compared to the mock GFP control cells, supporting that pluripotency is supported in these cells. Culturing GFP-N2KASH/N1KASH longer without LIF for 7 days showed (Figure 3-4 and Figure 3-5) varied gene expression levels of Oct4, Nanog, Sox2, Rex1, Stat3 and lamin A, suggesting possible presence of spontaneous differentiating cells when the cells were cultured for longer period without LIF. Lamin A still showed lower expression in dominant negative KASH domain of Nesprin 1 and 2 compared to the mock GFP cells.

Nesprin 1 and Nesprin 2 are widely expressed in cells with a variety of splice variants. The nesprins link the cytoskeleton to the nucleus by their association with the nuclear envelope LINC.
complex (Mellad et al., 2011; Khatau et al., 2012; Starr, 2011; Alam et al., 2015, Zhang et al., 2005; Ramdas and Shivashankar, 2015, Arsenovic et al., 2016).

Previous works showed that both Nesprin 1 and 2, along with other members of the LINC complex, facilitate nuclear shape, endothelial cell shape, cell migration and cell architecture (Rajgor et al., 2012; Zhang et al., 2001; Warren et al., 2005; King et al., 2013). A study also reported that increased expression of Nesprin 1 aids the changes to structure of the nuclear envelope during stem cell differentiation (Smith et al., 2010). In this work, I have shown for the first time that Nesprin 1 and Nesprin 2 are directly involved in the regulation of mouse ESC pluripotency. Using dominant negative KASH domain experiments, I compared the impact of overexpressing KASH domain Nesprin 2 with the overexpression of the domains of both Nesprin 1 and Nesprin 2. Interestingly, the simultaneous overexpression of KASH domains of Nesprin 1 and 2 had a more significant impact on the ESC pluripotency enhancement compared to the domain of Nesprin 2 alone (Figure 3-4). This suggests the different roles of Nesprin 1 and 2 in regulating the pluripotency of mouse ESCs. However, in this study, the downregulation of Nesprin 1 by siRNA in ESCs could not be used to verify if Nesprin 1 silencing affects pluripotency since the addition of the transfection drug alone is sufficient to enhance ESC pluripotency (Figure 3-7). The increased membrane permeability to DNA caused by the transfection drug appears to positively affect the pluripotency of ESCs.

Zhang and colleagues reported that compared to Nesprin 1, Nesprin 2 has a reduced impact on the nuclear morphology in primary human dermal fibroblasts and human bone osteosarcoma cells (Zhang et al., 2007). Additionally during myogenesis, human myoblasts have increased
Nesprin 1 expression compared to Nesprin 2 (Randles et al, 2010), suggesting the different roles of Nesprin 1 and Nesprin 2 in different cells.

This work highlights the importance of F-actin filament connection to the nucleus in regulating ESC pluripotency. Lamin A is suggested to be a contributor to the stiffness of the nucleus (Isermann and Lammerding, 2013). A previous study reported that undifferentiated and differentiated cells have very different nuclear shapes, plasticities and mechanical stiffness (Khatau et al., 2012). The ablation of Nesprin 1 and 2 generate different nuclear position, shape and chromatin positioning (Banerjee et al., 2014). It is possible that in differentiated cells, higher expressions of lamin A account for the structural changes in the nuclear envelope that eventually inhibit the pluripotency genes. Overexpression of dominant negative KASH domain of Nesprin 1 and 2 showed higher levels of ROCK2 (Figure 3-3). Rock inhibits actin depolymerization (Bhadriraju et al., 2007), this result suggests that in the dominant negative cells, ROCK is upregulated to increase actin polymerization as a means to compensate for the loss of actin-nuclear connection by the nesprin. In the disruption of Nesprin 1 and Nesprin 2, the reduced direct physical connection between the substrate and the ESC nucleus via F-actin filaments and the cell membrane, preserves the nucleus from the effects of extracellular mechanical stress and signals, thus allowing it to maintain the expression of its pluripotency factors.

The findings in this chapter suggest that lowering cellular levels of Nesprin 1 and Nesprin 2, can support the maintenance of the pluripotent state of the ESCs. The detailed understanding of the underlying mechanism of these proteins will help to address the issues related to stem cell maintenance and disease related research.
Chapter 4 General discussions

Pluripotency is defined as the capability of cells to produce identical daughter cells that can differentiate into almost any cell type of the organism. The unique capability of stem cells to self-renew indefinitely offers a valuable approach for cell-based therapies and regenerative medicine. The stable expressions of transcription factors Oct4, Sox2 and Nanog are known to sustain the pluripotency of ESC (Nichols et al, 1998; Boyer et al., 2005; Loh et al., 2006; Marson et al., 2008). These factors are regulated by signaling pathways connected with the extracellular environment. However, even in the absence of extracellular signals, due to the intrinsically stochastic nature of the pluripotency network, stem cells often display heterogeneous gene expressions, resulting in spontaneous differentiation of the cells. As a result, in ESC cultures, the leukemia inhibitory factor (LIF) cytokine is often used to suppress cell differentiation (Smith et al., 1988; Williams et al., 1988).

Studies on transcriptional regulation of ESC have mostly focused on using biochemical signals, such as LIF, to maintain pluripotency. However, recent works have shown the importance of mechanical signals from ESC microenvironment in regulating its fate. In the first part of this dissertation, I successfully inhibited the spontaneous differentiation of mouse ESC in the absence of LIF by culturing the cells on soft substrates. The work further revealed that actin filaments are activated in response to mechanical stress from rigid substrates, which eventually results in the loss of pluripotency. It also suggests that mechanical signal is transmitted from cell substrate to nucleus through F-actin. When F-actin disruption was observed in response to soft substrate, pluripotency of the cells is supported.
In the final part of this dissertation, I showed that disrupting the actin cytoskeletal network connection to the nucleus directly by overexpressing the dominant negative KASH domain of Nesprin 1 and Nesprin 2, supported the pluripotency of ESC on rigid substrates in the absence of LIF up to day 7. The reduced direct physical connection between ESC nucleus and the cell membrane could be isolating the nucleus and its morphology from the effects of extracellular mechanical signals and stress, and thus allowing the nucleus to maintain the expression of its pluripotency factors (Figure 4-1).

Concluding remarks and future perspectives

The pluripotent stem cells remarkable potential to self-renew and to differentiate into almost all cells in the body makes them significant for functional tissue engineering research and cell-based therapies. For successful therapeutic applications of ESC, it is crucial to improve ESC culture by understanding the mechanisms of pluripotency maintenance. In this work I have revealed that mechanical factors can take over the role of LIF biochemical signals in maintaining the pluripotency of ESC in culture. I have shown that the collapse of focal adhesion due to the absence of mechanical stimuli from the soft substrates enhances the pluripotency factors in ESC. Moreover, the direct disruption of actin cytoskeletal connectivity to the nucleus in ESC also enhanced the pluripotency factors. This work demonstrates the importance of intracellular signal transmission from focal adhesion to the nucleus in the regulation of pluripotent gene expression in mouse ESCs. Specifically, it shows that extracellular mechanical signals can control the pluripotency of ESC via the actin cytoskeleton – nucleus connectivity. To continue the research in this work, experiments such as the stable cell line with Nesprin 1 and 2 knockdowns can further
evaluate the findings from this work. Furthermore, single-molecule analysis of core transcription factors, Nanog could also be evaluated in the knockdown experiment. To support the finding from Chapter 2, knockdown experiment of ROCK2 could also further support the finding from this work.

**Figure 4-1** Schematic of the summary of the findings in the study
References


List of Publications

Publications


Poster/Presentation

Acknowledgements

I would like to thank my supervisor Professor Tomonobu Watanabe for accepting me as his lab member. I appreciate his patience, teachings, encouragement and advice throughout my time in the lab. His friendly nature encouraged me to consult him whenever I needed help. I am also thankful to him for providing financial support toward the conclusion of my studies. I am grateful to my adviser, Professor Hideaki Fujita for introducing me to the world of mechanobiology, and for providing insightful ideas in research. He has guided me closely during journal paper preparations.

I am indebted to Professor Toshio Yanagida for the opportunities available at the RIKEN Quantitative Biology Center (QBIC) to expand my research experience and for his advice. I would like to acknowledge my dissertation committee members, Professor Yasushi Hiraoka, Professor Tatsuo Fukagawa and Professor Masahiro Ueda for their comments and guidance during my defense. My conversations with Professor Hiraoka and his crucial suggestions helped me to strategize my research and gave me the confidence to complete my dissertation.

I thank the RIKEN International Program Associate initiative for the financial support during my studies, which allowed me to work among leading scientists. I learned good research ethics and work habits from these colleagues.

I highly appreciate Professor Sayaka Higuchi, Professor Hiroaki Machiyama, Dr. Akira Takai, Dr. Taro Ichimura, Dr. Junichi Kaneshiro, Dr. Takamitsu Morikawa, Dr. Ryota Mizushima and Mr. Kazuki Matsuda, for guiding me well and patiently when I was performing experiments and for their valuable technical advice.
The thoughtful advice given by Dr. Kazuko Okamoto, Dr. Arno Germond, Mr. Taishi Kakizuka and Ms. Keiko Yoshizawa have been crucial in my research. A special thank you to Ms. Yulia Panina for her guidance in experimental techniques, insightful views and our little talks about the mouse experiments.

I appreciate the kind assistance provided by the administrative personnel of Osaka University and RIKEN QBiC, Ms. Yuko Yoshimura, Ms. Ai Tsujita, Ms. Yasuko Kakizuka, Ms. Eri Taniguchi and especially Ms. Ayako Lorens. They have always supported me and helped me to fulfill my responsibilities as both Osaka University graduate student and an International Program Associate in QBic.

I came to know Mrs Satoko Noda on a train ride but our friendship quickly turned into a very special one because of the genuine affection she showed me and especially toward my son. Despite being octogenarian, she would visit us often, always bringing by some nice food! Her friendship makes me appreciate the beautiful culture of Japan. I am fortunate to have had the opportunity to study and live here.

At the beginning of my graduate studies, the apprehension of continuing my education in a new field after a long break quickly dissolved because of the kind friendship and support I received from my course mates Dr. Menglu Li and Ms. Emilda Gomez. I cherish their warmth, passion, determination and their deep concern. I am grateful to have them as my best friends.

I am grateful to my family for their continuous support, care and concern. My parents' blind belief in me motivated me into pursuing graduate studies. I am forever grateful to my sister Diana and
admire her compassion and confidence. My brothers and sisters are my fortress and I always cherish their presence.

Seventeen years ago, I met a man whose distinctive views on life, determination, and empathy, mesmerized me. Having stuck with me since then, Satya's passion and fortitude continues to inspire me. He has always been supportive of my studies and have been a pillar during my enduring times. He is my best friend.

I cherish the laughter, the happiness and the unconditional love of my Aritya. He is my source of inspirations and joy, and the reason for me to strive forward.

I thank you all.