The Role of Nitric Oxide Signalling on Direct Reprogramming of Adult Stem Cells and Fibroblasts

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List of Abbreviations

3D: Three dimensional
ALP: Alkaline phosphatase
CAV-1: Caveolin-1
CAV-1^{F92A}: F92A mutation encoded caveolin-1
CSD: Caveolin-1 scaffolding domain
DAF-2DA: 4,5-diaminofluorescein diacetate
DMEM: Dulbecco’s Modified Eagle Medium
DNA: Deoxyribonucleic acid
DNMT: DNA methyltransferase
DOX: Doxycycline
eASC: Equine adipose-derived stem cell
ECM: Extra cellular matrix
ESC: Embryonic stem cell
FBS: Fetal bovine serum
FGF2: Fibroblast growth factor 2
FGFR: fibroblast growth factor (receptor)
GATA4: GATA binding protein 4
GFP: Green fluorescent protein
H&E: Haematoxylin & Eosin
HDAC: Histone deacetylase
iPSC: Induced pluripotent stem cell
L-NAME: L-N^G-nitroarginine methyl ester
LB: Luria Bertani
Lv: Lentiviral vector
MC: Minicircle
MEF2C: Myocyte-specific enhancer factor 2
MYH6/α-MHC: Myosin heavy chain α isoform
MMLV: Moloney murine leukemia virus
MSC: Mesenchymal stem cell
NBF: Neutral-buffered formalin
NO: Nitric oxide
NOS1/nNOS: Nitric oxide synthase isoform 1 or neuronal nitric oxide synthase
NOS2/iNOS: Nitric oxide synthase isoform 2 or inducible nitric oxide synthase
NOS3/eNOS: Nitric oxide synthase isoform 3 or endothelial nitric oxide synthase
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PDGFR: Platelet-derived growth factor (receptor)
pDNA: Plasmid DNA
PKB/Akt: Protein kinase B
rBMSC: Rat bone marrow-derived mesenchymal stem cell
RNA: Ribonucleic acid
RT-qPCR: Reverse transcription quantitative polymerase chain reaction
SD: Standard deviation
SEM: Standard error of mean
TBX5: T-Box 5
TE: Terrific broth
TGFβ: Transforming growth factor beta
VEGF: Vascular endothelial growth factor
VEGFR: Vascular endothelial growth factor (receptor)
WT: Wild type
Certificate of Authorship

I hereby declare that this submission is my own work and to the best of my knowledge and belief, understand that it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged.

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Thesis Structure

This PhD thesis is divided into eight chapters that include five research chapters. Chapter 1 is a literature review and hypothesis. Chapter 2 presents general materials and methods. Chapters 3 to 7 are research chapters in which two of them (chapter 3 and chapter 6) were published. Published articles are attached as appendix 3 and 5. Chapter 8 presents general discussion, conclusions and future directions.
List of Research Articles


Book Chapters

List of Conference Articles

Oral presentations


Poster presentations


Abstract

Degenerative diseases of the musculoskeletal, cardiovascular and gastrointestinal system are common health issues worldwide and current treatments are inefficient. In this context, cell therapy based approaches have attracted much interest, however, a number of improvements are required to enhance their therapeutic efficiency in particular promoting specific cellular differentiation events at the site of transplanted cells such as in bone fractures, myocardial infarction and chronic wounds. Genetic modification of adult stem cells is a promising approach to induce particular signalling pathways which may drive cellular differentiation to specific cell types. Nitric oxide (NO) is an important molecule which can interact with various alternative signalling pathways to promote cellular differentiation into a number of different cell types. NO is a pleiotropic diffusible gas and plays important roles, including maintenance of vascular tone, blood pressure regulation, bone and cardiovascular development and promotion of neo-angiogenesis. Endothelial nitric oxide synthase (eNOS/NOS3) is one three enzymes responsible for producing NO, and it is located in specialized cell membrane structures known as caveolae and is held in a tight conformation with caveolin-1 (CAV-1WT) protein. This interaction inhibits NO production and is relaxed during physiological shear stress allowing NO release. CAV-1WT acts as a scaffolding protein regulating eNOS activity, recent research has shown that mutations within the eNOS interacting scaffolding domain of CAV-1 (CSD; amino acids 82-101), in which substitution of phenylalanine (F) at
the amino acid position 92 to alanine (CAV-1^{F92A}) removes CSD mediated eNOS inhibition and increases eNOS mediated NO release. This thesis describes the effects of NO on a variety of cellular differentiation programs. eNOS-NO signalling was reconstituted in mesenchymal stem cells (MSCs) and fibroblasts using lentiviral vector technology. To increase eNOS derived NO release, eNOS and CAV-1^{F92A} were co-expressed, which could significantly increase NO production in all modified cell types. Increasing NO availability in equine adipose derived stem cells through modification with lentiviral vectors to express eNOS alone (eASC^{eNOS}) or eNOS+CAV-1^{F92A} (eASC^{eNOS+CAV-1^{F92A}}) induced osteogenic differentiation (chapter 3) as evident by increased calcium deposition and osteogenic specific markers, Runx2 and Alp gene expression and the activity of a Runx2-eGFP reporter. Inhibition of eNOS through treatment of 2 mM L-NAME resulted in reduced Runx2, and Alp expression confirming that enhanced osteogenesis was NO mediated and this NO mediated osteogenesis was driven by endogenous regulation of Wnt/β-catenin signalling, by up-regulating canonical Wnt signalling and, inhibiting non-canonical Wnt signalling. This relationship between NO and enhanced osteogenic differentiation of adult stem cells is a novel finding. In chapter 4, the co-expression of eNOS and CAV-1^{F92A} in rat bone marrow MSCs (rBMSC^{eNOS+CAV-1^{F92A}}) could significantly enhance endothelial differentiation through enhancing Wnt/β-catenin signalling, and the increased levels of NO could specifically direct rBMSC differentiation towards arterial endothelial cells (ECs), in which NO could promote arterial specific Notch1, Dll4 and Hey2 gene expression whilst venous specific Coup-tflII and lymphatic specific Prox1 master transcription
factors were down regulated. In addition, the expression of DNA methyltransferase (*Dnmt1*) and histone deacetylase (*Sirt6*) were down regulated in rBMSC*eNOS*CAV-1F92A cells suggesting that NO may regulate epigenetic modification to promote endothelial differentiation. Demonstrating the vessel formation ability of genetically modified cells, subcutaneous transplantation of the rBMSC*eNOS*CAV-1F92A cells seeded in polyurethane scaffolds in rats resulted in formation of blood vessels. Chapter 5 of this thesis analysed the effect of increased levels of NO through co expressing of eNOS and CAV-1F92A in human foreskin fibroblasts, BJ (BJ*eNOS*CAV-1F92A) on GMT mediated cardiac reprogramming and this concept has not been reported previously. Cardiac reprogramming of human fibroblasts, through expression of Gata4, Mef2C, and Tbx5 transcription factors (termed as GMT) has been shown to increase cardiomyocyte generation, however the efficiency is reported to be low. Reconstitution eNOS-NO signalling could significantly enhance GMT mediated cardiac reprogramming, possibly through induction of *WNT3A* expression. FACS analysis of cardiac specific cTNT expression, demonstrated that only BJ*GMT*eNOS*CAV-1F92A cells were positive for cTNT (0.1%) and treatment of BJ*GMT*eNOS*CAV-1F92A cells with the eNOS inhibitor, 2 mM L-NAME reduced cTNT expressing cells suggesting that cardiac reprogramming of BJ was NO mediated. Furthermore, BJ*GMT*eNOS*CAV-1F92A cells could significantly induce atrial specific, *MYH6* and *MYL7* gene expression whilst ventricular specific *MYH7* and *MYL2* genes were not detected. On the other hand, over expression of eNOS and CAV-1F92A together with GMT could reprogram BJ into cardiomyocytes-like cell with binuclear morphology suggesting that increased
levels of NO may drive cardiac reprogramming of BJ into distinct type of cells. In chapter 6, a novel non-viral vector system capable of delivering a therapeutic gene to rBMSCs, was developed. A minicircle (MC) vector expressing eNOS (MC-eNOS) was constructed which increased gene delivery efficiency (21 ± 3 %) to rBMSCs was achieved compared to a conventional plasmid vector (9 ± 1 %) (P-eNOS) and also resulted in higher nitrite levels in MC-eNOS transfected cells. Furthermore, a therapeutic effect of eNOS delivery through the MC vector was seen through enhanced, in vitro capillary tubule formation in MC-eNOS transfected cells compared to P-eNOS and gene expression analysis showed, up regulation of angiogenic responsive genes, Vegfa and Fgf2 and their corresponding receptors Pdgfra and Fgfr2. The endothelial-specific marker Cd31, also was significantly increased in MC-eNOS-cells indicating conversion of rBMSCs to endothelial lineage. In chapter 7, to investigate further potential new mutants of caveolin-1 which may contribute to enhance NO production, a series of mutants of the CAV-A subdomain were constructed. Using an Alanine scanning approach combined with bioinformatics analysis, Ile-84 substitution to alanine (I84A) were predicted to have lowest hydrophobicity, and mutants (D82A, G83A, and I84A) were generated using site directed mutagenesis approach. Lentiviral vectors expressing these mutant caveolin transgenes together with F92A (CAV-1F92A-D82A, CAV-1F92A-G83A, CAV-1F92A-I84A) or CAV-1I84A, and eNOS were delivered to HEK293T cells and nitrite levels was measured. No significant increase in nitrite levels was associated with these new CAV-A domain mutants highlighting the uniqueness of the F92A mutation in terms of eNOS mediated NO release.
In summary, the data presented in this thesis highlights the potential to promote cellular reprogramming through enhancing NO production using a mutant caveolin-1 transgene to reduce inhibition of eNOS activity. This approach may provide a novel platform for gene modified cell based regenerative medicine strategies.
Chapter 1: Literature review
1.1. Stem cells

Stem cells can be broadly described as a group of undifferentiated cells capable to self-renewal (cell division without differentiation) and can subsequently differentiate into specialized cells types (Morrison and Kimble, 2006; Weissman et al. 2001). Stem cell division can be described as symmetric and asymmetric (Morrison and Kimble, 2006). Symmetric cell division yields two daughter cells showing the same characteristics of the parent stem cell and has potential to differentiate into other lineage cell types (Morrison and Kimble, 2006). On the other hand, asymmetric cell division yields differentiated cells through development of lineage specific intermediate progenitor cells (Knoblich 2008). Progenitor cells are generated as an intermediate state before the stem cell is converted into the fully differentiated cell type (Weissman et al. 2001) and are regarded as being committed to differentiating along a particular cellular developmental pathway. There are two types of resident stem cells, which are categorized as embryonic stem cells (ESCs) or somatic / adult stem cells. ESCs are referred to as pluripotent, an ability to differentiate into all the cell types in the body, whereas adult stem cells are multipotent and demonstrate a restricted ability to differentiate into multiple lineages.
1.2. Embryonic stem cells

Embryonic stem cells (ESC) are a class of unspecialized cells derived from the inner cell mass of a blastocyst which as an early stage of the embryo containing 200 to 250 cells (Thomson et al. 1998 and Reubinoff et al. 2001). ESCs are pluripotent stem cells, which can differentiate into any cell type represented within three germ layers (mesoderm, ectoderm, and endoderm) (Keller et al. 2005). In response to various stimuli during development, ESCs can be differentiated into specialized cells which have specific roles in the body (Takasato et al. 2014, Soh et al. 2016). There are two key features which characterize ESCs, are, pluripotency (the ability to differentiate into all three germ layers, ectoderm, endoderm and mesoderm) and self-renewal (the ability to go through numerous cycles of cell division while maintaining the undifferentiated state) whereby they are maintained as pure populations of undifferentiated cells in culture for extended periods of time, retaining a normal karyotype unlike tumor cell lines (Keller, 2005). Over the past two decades, ESCs have been using as a model system for studying the basic processes in mammalian development, and cellular differentiation events (Huch et al. 2015). ESCs have also provided a valuable platform for regenerative medicine and tissue engineering for development of future treatments of human diseases. Furthermore, ESCs have been also used as a reference in vitro model for understanding key molecular mechanisms which control cell fate and organogenesis (Davidson et al. 2012).
1.2.1. Genetic makeup of ESC

Transcription factors play a key role in ESC biology and, ESC specific transcription factors such as OCT4 (octamer-binding transcription factor 4) (Rosner et al. 1990 and Okamoto et al. 1990), SOX2 (Sry box-containing gene 2) (Avilion et al. 2003, Masui et al. 2007), and NANOG (Chambers et al. 2003) have been identified as important for maintaining pluripotency. On the other hand, up regulation of cMYC (Cole et al. 1986, Dang et al. 1995) and KLF4 (Jiang et al. 2009) has been found to be important for long term maintenance of ESCs. Inactivation of, OCT4, SOX2 and NANOG promotes ESC differentiation to specialized cell types (Wang et al. 2012). Furthermore, interaction between SOX2 and OCT4 drives pluripotent specific gene expression such as NANOG (Rodda et al. 2005), in which OCT4 and SOX2 interact with the NANOG promoter which contains a highly conserved SOX-OCT cis-regulatory element essential for NANOG transcription. It is also documented that NANOG and SOX2 interact to regulate ESC self-renewal (Gagliardi et al. 2013). Using a chromatin immunoprecipitation approach on mouse and human ESCs, Rodda et al demonstrated that the Nanog promoter contains OCT4 and SOX2 binding sites (Rodda et al. 2005). Furthermore, Pereira and colleagues clearly demonstrated that repression of NANOG expression by TCF3 promoted ESC differentiation into mesoderm and endoderm (Pereira et al. 2006). Therefore, these ESC specific transcription factors repress gene expression essential for ESCs differentiation into different specialized cells and tissues indicating, OCT4, SOX2 and NANOG are the master regulators in ESC in which they maintain ESC features.
1.2.2. Limitations of ESC research

The use of ESCs in cell therapy applications is currently limited due to several factors such as 1) Ethical concerns associated with ESC isolation, 2) Immune rejection of allogeneic transplanted cells and 3) Teratoma formation following ESC transplantation due to accumulation of undifferentiated cells (Nussbaum et al. 2007).

1.3. Induced pluripotent stem cells (iPS cells)

To find an alternative pluripotent cell type to ESCs, in 2006, the Japanese scientists Shinya Yamanaka and Kazutoshi Takahashi demonstrated the groundbreaking discovery of induced pluripotent stem cells (iPSCs). iPSC cells are artificially created embryonic like stem cells generated by over expressing four transcription factors in somatic cells such as fibroblasts (Takahashi and Yamanaka, 2006). These iPSC cells exhibited similar features to ESCs. Since iPSC cells are artificially created cells, they do not have ethical and immunological problems associated with ESCs. Therefore, iPSC cells show potential in cell biological research, including their application in cell therapy, drug screening and disease modeling.
1.3.1. Generation of iPS cells

Differentiated cells can be reprogrammed into a pluripotent state by transfer of nuclear contents into oocytes (Wilmut et al. 1997) and, fusion of somatic cells with embryonic stem (ES) cells can also result in reprogramming to a pluripotent state (Cowan et al. 2005). These studies revealed that oocytes and ES cells contain factors which may be responsible for conversion of somatic cells to a pluripotency state. In 2006, Shinya Yamanaka and Kazutoshi Takahashi demonstrated that, mouse embryonic or adult fibroblasts can be reprogrammed back to an embryonic like state by overexpression of four transcription factors, OCT4, SOX2, KLF4 and cMYC (Takahashi and Yamanaka, 2006). They named these ES like reprogrammed cells as induced pluripotent stem cells (iPSCs). In 2007, the same investigators, demonstrated generation of iPSCs from human fibroblasts (Takahashi et al. 2007). Yu and colleagues have also reported generation of human iPSCs from fibroblasts with a slightly different combination of transcription factors, in which KLF4 and cMYC were replaced with NANOG and LIN28 (Yu et al. 2007). Both of these iPSC cells exhibited similar features to ES cells including morphology, proliferation, ESC specific gene expression profiles, and teratoma formation. This method of cellular reprogramming has been shown to be universal and can be applied to a variety of cell types such as B-cells (Wada et al. 2011), liver cells (Aoi et al. 2008), and umbilical cord blood mononuclear cells (Wang et al., 2013). Moreover, iPSC cells have been generated from different species such as monkey (Liu et al. 2008), rat (Takenaka-Ninagawa et al. 2014) and horse (Lepage et al. 2016).
1.3.2. Limitations of iPS cells in clinical applications

Even though iPS cells have provided a solution for many of the obstacles raised with ESCs, iPS cells also have inherent disadvantages in terms of clinical applications which include teratoma formation (Hong et al. 2009) and use of the oncogene cMYC as a reprogramming factor which can lead to tumorigenesis (Okita et al. 2007). Of note, some labs have reported the generation of iPS replacing cMYC with NANOG (Yu et al. 2007).

A second issue associated with the therapeutic application of iPS cells is their immunogenicity. Transplanted iPS cells have been considered to be immune tolerant by the recipient. However, induction of T-cell-dependent immune response in recipients has been demonstrated (Zhao et al. 2011).

1.4. Adult stem cells

Adult stem cells or somatic stem cells are multipotent stem cells which can be found in specific cellular niches of organs and tissues. Adult stem cells are essential for maintaining the health of organs throughout a life time (Yin et al. 2006). Somatic stem cells were first identified about 40 years ago with the discovery of hematopoietic stem cells (McCulloch and Till, 1960) and bone marrow stem cells (mesenchymal stem cells) (Friedenstein et al. 1968). Adult stem cells can be found in many tissues such as brain (Gonzalez-Perez, 2012), liver (Fiegel et al. 2006), heart (Zhang et al. 2015) and lung (Schilders et al. 2016) and adipose (Bunnell et al. 2008). Adult stem cells are multipotent, they can self-renew and
differentiate to all the cell types in their tissue and as well as other lineages such as cardiomyocytes (Li et al. 2015) neuron (Petersen et al. 2015) and endothelial cells (Zhang et al. 2016).

The use of adult stem cells in cell therapy applications is currently limited due to several factors such as 1) Limited differentiation potential (Bang et al. 2016), 2) The results obtained in animal models may not be directly translated to humans (Bang et al. 2016), 3) Loss of proliferative capacity under standard culture conditions as well as the method for the delivery of adult stem cells to the patient may impact on their ability to survive post transplantation (Li et al. 2016; Bang et al. 2016).

1.5. **Mesenchymal stem cells**

Mesenchymal stem cells (MSCs) or mesenchymal stromal cells were discovered by the Friedenstein et al in 1968 (Friedenstein et al. 1968). He observed a number of different types of cells in bone marrow cultures some of which were adherent to tissue culture plastic, showed fibroblastic morphology and formed colonies. These cells were named as colony forming unit fibroblasts, CFU-F (Lanotte et al. 1981) and were found to differentiate into bone, adipose, cartilage and muscle tissue. Caplan coined the term “mesenchymal stem cells” (MSCs) (Caplan, 1991) and MSCs obtained from human bone marrow aspirates were characterized (Pittenger et al. 1999). The BM aspirate was first separated by density gradient separation and plated on tissue culture plastic and the attached cells were counted based on
their colony formation ability. Approximately, 0.001 to 0.01% cells of total cells were found to be MSCs and expressed CD29, CD90, CD71 and CD106 surface markers and were negative for CD45, CD14 and CD34. Importantly, they found that, these cells were able to undergo 40 population doublings in vitro over 10 weeks.

By now, it has been able to isolate MSCs from many other sources such as adipose tissue, umbilical cord blood, and placenta and even from dental pulp. Researchers are interested to find stem cells from different organs especially focusing on strategies to repair the same organs with autologous stem cells. Interestingly, these studies demonstrated that, MSCs from different niches showed different CD marker expression, phenotype and population doublings. Whilst no definitive single surface marker for MSCs had been described so far, an internationally accepted set of criteria has been established by the International Society for Cellular Therapy (ISCT) (Dominici et al. 2006). Accordingly, ISCT outlined that, MSCs should be positive for CD73, CD90 and CD105, negative for CD19, CD34, CD45, CD11b and HLA-DR. In addition, they should attach to the plastic tissue culture plates and demonstrate an ability to differentiate to adipocytes, chondrocytes and osteoblasts in vitro.

For cell therapy applications, MSCs are remarkable since they show antiapoptotic and immunomodulatory features providing them non-immunogenic properties. MSCs release a variety of cytokines. Therefore, when MSCs were injected to the damaged tissues (Kidney) in animal models, they were able to reduce the
apoptotic rate of the surrounding cells, which was mediated by the secretion of several growth factors like VEGF, FGF2 and TGF-β from MSCs applied especially in hypoxic conditions (Togel et al. 2007, Parekkadan et al. 2007, Block et al. 2009). More specifically, they showed that infusion of MSCs is important for re-vascularization to recover from acute kidney injury in mouse through secretion of growth factors. Furthermore, proliferation of T-cells was inhibited when co-cultured with MSCs in vitro (Di Nicola et al. 2002, Krampera et al. 2003, Le Blanc et al. 2003). In addition to T-cells, other immune responsive cells such as natural killer cells, B-cells and immature dendritic cells also were affected by MSCs (Corcione et al. 2006; Sotiropoulou et al. 2006; Spaggiari et al. 2008). In particular, MSCs can inhibit B-cell proliferation by inhibiting the G0/G1 phase through release of paracrine factors that can inhibit B-cell differentiation through the inhibition of IgM, IgG, and IgA production. The immunomodulatory effects of MSCs on T-cells and NK cells have been shown to be driven by cytokines like TGF-β, PGE2 and IL10 (Gonzalez-Rey et a. 2010; Tasso et al. 2012; Nemeth et al. 2010; Qu et al. 2012; Spaggiari et al. 2008).

Thus, MSCs are important candidates for cellular therapies as they feature the characteristics like, (1) Their well-defined markers enable isolation with a high degree of purity, (2) Easy to expand in vitro to high numbers without losing their properties, (3) They can differentiate into different cell types, (4) They can migrate to the injured site in vivo, (5) They have immunosuppressive effects that contribute to their possible use in allogeneic grafting (Corcione et al. 2006; Sotiropoulou et al. 2006; Spaggiari et al. 2008).
1.5.1. **Clinical trials using mesenchymal stem cells**

In the literature, there are many studies investigating the regenerative capacities of MSCs in different disease models generated by employing different non-human animal species. Cardiac regeneration, liver regeneration, kidney regeneration, autoimmune diseases and graft versus host disease (GvHD), neurological diseases, pulmonary diseases, osteogenic diseases, and cartilage repair are the most widely studied conditions. Moreover, MSCs are also being investigated extensively by clinical trials, mostly in United States, Europe and East Asia, with trials investigating MSC use in neurological, liver, bone, heart diseases, GvHD and some autoimmune diseases such as diabetes (Tseng et al. 2008, Horwitz et al. 1999, Williams and Hare, 2011; Premer et al. 2015). In the following section, the clinical application of MSCs will be discussed and a particular attention will be given to their role in heart disease.

1.5.1.1. **Mesenchymal stem cells in bone repair**

Despite advances in orthopedic surgery, fracture nonunion remains a major problem. The ability of MSCs to differentiate into osteoblasts and chondrocytes has allowed them to be applied in orthopedic settings. During normal fracture healing, MSCs are proliferating, and differentiating into osteoblasts, and form bone, thereby repairing the injury (Tseng et al. 2008). However, some of the fractures can fail to heal properly and result in either delayed union or nonunion. Fractures which fail to heal after 6 to 8 months of treatment are considered nonunion.
Application of MSCs for fracture repair has been investigated in preclinical animal models. To this end, autologous bone marrow-derived MSCs have been seeded on ceramic cylinders to be implanted into fractures in rat femora and have observed successful bone formation 8 weeks later (Kadiyala et al. 1997). Many other preclinical studies have been performed with implantation of autologous MSCs and have shown successful bone regeneration (Arinzeh et al. 2003, Bruder et al. 1998, Kon et al. 2000, Viateau et al. 2007).

In clinical settings, an early study has shown that culture-expanded autologous MSCs seeded on ceramic scaffolds were able to repair large bone defects (Quarto et al. 2001). Furthermore, several important clinical studies have used autologous MSCs for repairing large bone defects including avascular necrosis which is also called as bone infarction or bone necrosis. Avascular necrosis of the femoral head is a serious bone disease in young individuals (Jones et al. 2016). For these patients, local application of MSCs have resulted in complete fracture healing, and decreased necrotic lesions in cases of osteonecrosis (Quarto et al. 2001, Marcacci et al. 2007, Kim et al. 2009, Zhao et al. 2012).

Other than bone damage due to fractures, metabolic bone disorders such as osteoporosis (OP) and osteogenesis imperfecta (OI) also account for bone loss. The first clinical trial to treat OI has been performed by Horwitz and colleagues in 2002 (Horwitz et al. 2002; Table 1.1). They systemically infused allogeneic bone marrow MSCs to treat six children who had severe OI, and this resulted in increased bone mineral density and accelerated linear growth at 18–36 months
after treatment. Another study from the same group has investigated MSC therapy for treating OI and they have demonstrated that infused MSCs increased osteogenesis by the production of a soluble mediator (Otsuru et al. 2012; Table 1.1).

In addition to bone marrow MSCs, allogeneic fetal MSCs (fMSCs) have been also transplanted in two separate patients with OI (Le Blanc et al. 2005). In this clinical trial, a female fetus with multiple intrauterine fractures, diagnosed as severe OI was transplanted with allogeneic fetal MSCs at the 32nd week of gestation. At 9 months of age, bone histology showed a regular arrangement of bone trabeculae suggesting that allogeneic fetal MSC engrafted and differentiated into bone in a human fetus.

A second fetus with OI has been transplanted with fMSCs at 31 weeks of gestation and did not suffer any new fractures for the remainder of the pregnancy or during infancy (Gotherstrom et al. 2014). These two clinical studies revealed that prenatal transplantation of allogeneic fMSCs in OI appeared to be promising.

Table 1.1. Clinical trials of mesenchymal stem cell therapy in bone repair

<table>
<thead>
<tr>
<th>Reference</th>
<th>Disease</th>
<th>Phase</th>
<th>No. of patients</th>
<th>MSC source</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horwitz et al. 1999</td>
<td>OI</td>
<td>I</td>
<td>3</td>
<td>Allogeneic</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Horwitz et al. 2002</td>
<td>OI</td>
<td>I</td>
<td>6</td>
<td>Allogeneic</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Le Blanc et al. 2005</td>
<td>OI</td>
<td>I</td>
<td>1</td>
<td>Allogeneic</td>
<td>In utero transplantation</td>
</tr>
<tr>
<td>Otsuru et al. (2012)</td>
<td>OI</td>
<td>Unknown</td>
<td>9</td>
<td>Allogeneic</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Gotherstrom et al. 2014</td>
<td>OI</td>
<td>I</td>
<td>1</td>
<td>Allogeneic</td>
<td>In utero transplantation</td>
</tr>
</tbody>
</table>
1.5.1.2. Mesenchymal stem cells in vascular repair

The formation of new vessels is the cornerstone of successful cardiac repair. There are 3 mechanisms of postnatal neovascularization; (1) angiogenesis, (2) arteriogenesis, and (3) postnatal vasculogenesis (Asahara et al. 2011) where endothelial precursors originating from the BM and assemble to create new blood vessels. It is still under debate whether the observed increase in capillary density and tissue perfusion is caused by differentiation of MSCs to endothelial cells and vascular smooth muscle cells or because of secretion of paracrine mediators and generation of new pericytes (Williams and Hare, 2011; Premer et al. 2015). There is evidence that MSCs act as pericytes, perivascular cells that are essential for vascularization by stimulating endothelial cells to form tube-like structures, and subsequently vascular networks (Cai et al. 2009). Expression of MSC markers was also detected on the surface of native non-cultured perivascular cells suggesting that blood vessel walls harbor a reserve of progenitor cells that may be integral to the origin of the MSCs and other related adult stem cells (Crisan et al. 2008). In vitro, MSCs express α-smooth muscle actin and β-actin filaments (Davani et al. 2003) whereas in vivo studies has shown that MSCs express an endothelial phenotype that enhanced microvascular density in vivo (Psaltis et al., 2008). Despite this evidence, several groups suggest that only a small number of vessels contain donor cells. Therefore, it is proposed that release of proangiogenic and proarteriogenic factors from the MSCs that play the most important role in neo-
vasculogenesis (Gomes et al. 2013) and neo-angiogenesis (Chen et al. 2003). In this work by Chen and colleagues, there was a significant increase in the levels of VEGF and bFGF (basic fibroblast growth factor) in the MSC-treated animals leading to angiogenesis after intravenous injection into rats 24 hours after middle cerebral artery occlusion (MCAo). They have further shown significant increases in newly formed capillaries at the boundary of the ischemic lesion in rats treated with hMSCs compared with rats treated with phosphate buffered saline (PBS). Further supporting this theory, Markel and colleagues (Markel et al. 2008) showed that MSCs expressing low levels VEGF have significantly less cardio reparative capabilities. In this work, female adult rats have been subjected to ischemia-reperfusion injury and after the injury, VEGF knockout MSCs or normal MSCs have been infused into the coronary circulation. Following MSCs treatment, they observed that VEGF knockout MSCs significantly impaired myocardial function whilst normal MSCs improved it suggesting the importance of VEGF associated with the MSC benefit.

1.5.1.3. Neo-vessel formation
The process of neovessel formation is an important event both during embryonic development and also in adult tissues following injury such as ischemic infarction. Neovessels from the neighbouring normal tissues are needed to form the vessel network and restore the blood supply to the damaged tissues. Both ECs and SMCs are essential for the formation of blood vessels. However, the detailed mechanism of SMC migration and differentiation is not fully understood.
Until recently, it was accepted that vessels in adult ischemic tissues could only grow by angiogenic mechanisms, in which, the sprouting of mature ECs from pre-existing vessels was likely in response to angiogenic factors. However, recent studies have revealed that endothelial progenitor cells (EPCs) circulate postnatally in peripheral blood. These may be recruited from the bone marrow and incorporate into sites of active neovascularisation in ischemic hindlimbs, ischemic myocardium, injured corneas and tumor vasculature (Asahara et al. 1999). This process is termed postnatal vasculogenesis (Luttun et al. 2002). EPCs participate in vasculogenesis by differentiation into endothelial cells (ECs) and thereby promote angiogenesis through the production of angiogenic growth factors (Miyamoto et al. 2007). Accumulating evidences has shown that EPCs have a therapeutic potential for vascular repair through promoting the re-endothelialization of damaged vessel walls and the neovascularization of ischemic tissues (Rafii and Lyden, 2003; Kim et al. 2015).

Bone-marrow-derived mesenchymal stem cells (BMSCs) and multipotent adult progenitor cells (MAPCs) can be differentiated into endothelial-like cells in vitro and contribute to neoangiogenesis in vivo (Al-Khaldi et al. 2003; Reyes et al. 2002). Furthermore, BMSCs can increase collateral remodeling and perfusion in ischemic models through paracrine mechanisms rather than by cellular incorporation upon local delivery (Kinnaird et al. 2004; Cai et al. 2016a).

Recently, it has been shown that adult BMSCs, under appropriate in vitro environmental cues, can be induced to undergo vasculogenic differentiation
culminating in microvessel morphogenesis. When rat BMSCs were seeded onto a three-dimensional (3D) tubular scaffold, the maturation and co-differentiation into endothelial and SMC lineages which led to successful microvessel formation was observed (Valarmathi et al. 2009). A separate study showed that locally delivered, activated cardiac progenitor cells (CPCs), could generate coronary vasculature by dividing and differentiating in both ECs and SMCs, restoring blood supply to ischemic myocardium (Tillmanns et al. 2008).

1.5.1.4. Mesenchymal stem cells in cardiac repair

Ischemic heart disease is associated with the highest mortality rate among all diseases (http://www.who.int). There is an urgent need for alternative cell based therapies to treat cardiovascular diseases. Broadly, Ischemic heart diseases are characterized by a shortage of blood supply to the different regions of the heart resulting in these regions undergoing necrosis and apoptosis. With a limited endogenous regeneration available to the mammalian heart, heart transplantation is often the only therapeutic option currently available.

Cell therapy to regenerate damaged cardiac tissue is an exciting alternative to heart transplantation. In 1995, Wakitani et al reported that generation of cardiomyocytes in vitro from rat bone marrow derived MSCs (rBMSCs) (Wakitani et al. 1995). Following this, several studies reported the successful differentiation of MSCs into cardiomyocytes (Makino et al. 1999, Bittira et al. 2002). Both of these studies demonstrated the in vitro generation of beating cardiomyocytes from rat bone marrow MSCs.
A number of in vivo studies have been performed to investigate the efficacy of MSCs in cardiovascular regeneration. In 2002, Shake et al demonstrated that swine bone marrow derived MSCs could be differentiated into functional cardiomyocytes when injected into the infracted swine myocardium (Shake et al. 2002). On the other hand, when MSCs were injected intracardially in a canine model, the MSCs were differentiated into smooth muscle cells and endothelial cells (Silva et al. 2005) and further studies showed that, when MSCs were injected into a rat myocardial infarct, there was a significant reduction in the damaged area (Tang et al. 2006). Moreover, genetic modification of MSCs to overexpress Akt, exerted a beneficial effect (Lim et al. 2006, Mangi et al. 2003), suggesting that genetic modification of MSCs would provide a better platform for cardiovascular repair. It is possible that Akt may activate mammalian target of rapamycin complex 1 (mTORC1) and forkhead box o3 (Foxo3a) which are acted downstream of Akt to promote cardiomyocyte reprogramming (Zhou et al. 2015).

1.5.2. Direct MSC stimulation of endogenous repair

MSC transplantation to the heart has been shown by multiple groups to stimulate proliferation and differentiation of endogenous cardiac stem cells (Hatzistergos et al. 2010; Loffredo et al. 2011; Suzuki et al. 2011). Neo-myogenesis can be promoted by 2 related mechanisms through stimulation of endogenous cardiac stem cells (c-kit+ and other lineages such as cardiac fibroblasts) and enhancement of myocyte cell cycling (Hatzistergos et al. 2010). To demonstrate this, GFP+ allogeneic MSCs were injected into infarcted swine hearts and allowed to form
chimeric clusters of immature MSCs and endogenous c-kit+ cardiac stem cells. 
These clusters exhibited cell–cell interactions mediated by connexin-43 mediated 
gap junction formation and N-cadherin mechanical connections. Importantly, the 
endogenous c-kit+ cell population was increased by 20-fold in MSC-treated 
animals relative to controls, and furthermore the c-kit+ cells showed a high 
capacity for myocyte lineage commitment (Hatzistergos et al. 2010). It has been 
demonstrated that, when MSCs were co-cultured with rat ventricular myocytes, 
MSCs became actin-positive and formed gap junctions with the native myocytes 
(Xu et al. 2004). Furthermore, an improvement in myocardial wall thickening in pigs 
with hibernating myocardium which is a pathology when some segments of the 
myocardium exhibit abnormalities of contractile function, was induced upon MSC 
injection (Suzuki et al. 2011) compared with control. This same study also found a 
4-fold increase in c-kit+ and CD133+ populations that co-expressed Gata4 and 
Nkx2.5 at 3 days through 2 weeks in animals receiving MSCs. In a preclinical 
study, the combination of human MSCs and c-kit+ cardiac stem cells showed 
enhanced cardiac regeneration (Williams et al. 2013).

1.5.3. Preclinical trials of MSC
Toma et al showed that, human MSCs were differentiated to a cardiac fate when 
injected into murine hearts (Toma et al. 2002). In this study, MSCs labeled with 
lacZ, were injected into the left ventricle of adult mice, and after one week post 
injection, the lacZ labeled MSCs morphologically resembled the surrounding host 
cardiomyocytes and furthermore they expressed cardiac specific genes such as α- 
actinin, cardiac troponin T. It has been shown that MSCs can modulate host
immune responses when allogeneic porcine MSCs were injected \((2 \times 10^8\text{ cells})\) intramyocardially into 3-day-old immune-competent porcine infarcted hearts this resulted in long-term engraftment and a significant decrease in scar tissue without an inflammatory response (Amado et al. 2006). MSCs have also been tested in numerous cardiovascular settings. In a separate study, where porcine MSCs were injected endomyocardially of one of three MSC doses \((2.4 \times 10^7, 2.4 \times 10^8, 4.4 \times 10^8\text{ cells})\) into the porcine heart 5 days after infarction, an improvement in ejection fraction (EF) and a reduction in scar formation was seen in MSC-treated animals (Hashemi et al. 2008). The effect of MSC dosage was examined in ovine models of MI where different doses of ovine BM-derived MSCs \((2.5 \times 10^7, 3.75 \times 10^7, 5 \times 10^7\text{ cells})\) were directly injected into sheep hearts 1 hour post MI (Houtgraaf et al. 2013), and improvements in end diastolic volume were only seen in animals receiving the 2 lower doses, although the EF increased regardless of the cell dosage (Houtgraaf et al. 2013) suggesting that there may be a therapeutic threshold relating to the total number of cells that can be injected and a beneficial therapeutic outcome. In a study with a different species (dogs), chronic myocardial ischemia was produced by implantation of an ameroid constrictor in the proximal left anterior descending coronary artery (LAD) and diagonal branch ligation, followed by injection of allogeneic canine MSCs \((1 \times 10^8\text{ cells})\) into canine hearts resulted in increased EF, vascular density, and a decrease in scar tissue (Silva et al. 2005). Furthermore, it has been reported that region specific administration of allogeneic porcine MSCs \((2 \times 10^8\text{ cells})\) to the border and to infarct zones of porcine myocardium 3 days after MI also reduced scar size by 50% (Amado et al. 2006)
with improvements in EF, left ventricular end-diastolic pressure, relaxation time, and systolic compliance in the treated animals. Furthermore, in a model of acute myocarditis in rat attenuation of myocardial inflammation was attenuated when autologous rat MSCs (3 x 10^6 cells) were injected into 10-weeks-old rats (Ohnishi et al. 2007), together with increased capillary density in MSC treated animals.

1.5.4. Clinical trials of MSC based therapies for cardiac repair

1.5.4.1. Acute Myocardial Infarction

In a phase I randomized study, 53 patients received different doses of allogeneic human MSCs (0.5, 1.6, and 5.0 x 10^6 hMSCs/kg) 7 to 10 days post MI (Hare et al. 2009). The MSCs were injected intravenously. Six months after infusion, clinical data showed fewer arrhythmic events, and an improved EF. Following the success of this pilot study, a phase II trial was established to investigate whether allogeneic MSCs were as safe and effective as autologous MSCs in patients with left ventricular (LV) dysfunction due to ischemic cardiomyopathy (Hare et al. 2012). Upon intravenous infusion of allogeneic MSCs (2 x 10^7 cells) within 7 days of an acute MI, resulted in reduced cardiac hypertrophy, stress induced ventricular arrhythmia, heart failure, LV end-diastolic volumes and increased EF. Interestingly, allogeneic MSCs did not stimulate significant donor-specific alloimmune reactions. In a separate study, Chen and colleagues have injected autologous MSCs (1x10^{11} cells) intracoronarily in patients with subacute MI and observed decreased perfusion defects, improved left ventricular ejection fraction, and left ventricular remodeling 3 months after therapy (Chen et al. 2004a). Other clinical benefits
attributed to MSCs include decreased perfusion defects, improved left ventricular ejection fraction, and left ventricular remodeling when MSCs were administered to patients with subacute MI (Houtgraaf et al. 2012). In addition to bone marrow MSCs, adipose-derived MSCs have also been used to treat acute MI. A trial with 14 patients, which tested the safety of intracoronary injection of freshly isolated adipose-derived MSCs after myocardial infarction (Perin et al. 2014), demonstrated improved cardiac function, accompanied with a significant improvement in perfusion defect and a 50% reduction in myocardial scar formation.

There are a reported 41 clinical trials in which MSCs have been applied in relation to cardiac injury and repair between 2010 and 2015 (Singh et al. 2016). There is also an ongoing clinical trial using adipose-derived MSCs, in patients with chronic myocardial ischemia (Mushtaq et al. 2014) where they used culture-expanded adipose tissue derived MSCs. This study has been designed to investigate the safety and efficacy of intramyocardial delivery of VEGF-A165-stimulated autologous adipose tissue derived MSCs to improve myocardial perfusion and exercise capacity (Mushtaq et al. 2014). The list of completed and ongoing clinical trials has been summarized in appendix 1.

1.5.4.2. Phase III Clinical Trials

There are 6 ongoing phase III clinical trials using MSCs. Of note, one of these studies (Bartunek et al. 2013), applied autologous MSCs treated ex vivo with cardiogenic growth factors (TGF-β, BMP4, FGF2, cardiotrophin, α thrombin) to
enhance their commitment to the cardiopoietic lineage and investigators reported significant improvements in EF, and end-systolic volume compared with controls. Other phase III studies are currently underway, in which one in United States is treating 600 patients with chronic heart failure (https://clinicaltrials.gov/ct2/show/NCT02032004) all the phase III clinical trials currently undergoing are listed in the table in appendix 1.

1.6. Direct reprogramming of adult stem cells

With increasing use of MSCs in clinical trials, improving the ability of MSCs to become cells of interest has been the main focus of reprogramming. Genetic modification is one approach to convert adult stem cells from one developmental lineage to another is mainly achieved by overexpression of lineage restricted transcription factors, and various gene transfer methods have been used.

1.7. Integrating vectors

1.7.1. Delivery of reprogramming factors via retroviral vectors

Retroviral vectors are commonly used as gene delivery systems since they are well characterized and have high transduction efficiencies. For gene delivery approaches, replication defective viral vectors are used. In these vectors, coding regions for the genes necessary for additional rounds of virion replication and packaging are deleted. Viruses generated from replication-defective vectors can infect their target cells and deliver genes of interest, but avoid triggering the lytic pathway, which would result in cell lysis and death. Replication defective viral
vectors can usually package inserts of up to 10 kb. The major disadvantage of the retrovirus-mediated gene delivery approach is the requirement for cells to be actively dividing to allow transduction by the viral vectors. Thus, slowly dividing or non-dividing cells such as neurons are difficult to transduce efficiently with retroviruses. Stable integration of retroviral DNA into the host genome provides a platform for persistent expression of transgenes, however, this may lead to insertional mutagenesis, which is a phenomenon which can occur due to proviral integration into the host DNA. If proviral integration occurs within a transcriptional active region (gene) of the host genome, possible expression of endogenous oncogenes may occur. In a landmark study by Ieda et al, using a Moloney murine leukemia virus (MMLV) retrovirus-mediated gene delivery approach, it was demonstrated that mouse cardiac and dermal fibroblasts could be reprogrammed into cardiac muscle cells using three cardiac specific transcription factors, Gata4, Mef2C, and Tbx5 (Ieda et al. 2010). In this expression vector, expression of the transgenes was driven by the 5’MMLV long terminal repeat (LTR) promoter, which can be silenced by methylation (Takahashi and Yamanaka, 2006). This method has been used by several groups and the efficiency of reprogramming has been enhanced by using alternative transcription factors or small molecules (Summarized in the table 1.2 and table 1.3). The reprogramming efficiency of the retrovirus-mediated gene delivery approach is partially dependent on the stoichiometry of the delivered transcription factors (Wang et al. 2015). It was reported that a higher reprogramming efficiency can be achieved, greater than the original GMT experiment when the stoichiometry of the transcription factors is
changed (Wang et al. 2015). In this investigation, 6 different polycistronic lentiviral vectors were constructed to cover all possible combinations of G, M, and T with identical 2A sequences. Using this approach, it was demonstrated that each combination of G, M, T produced distinctly different G, M, T protein expression levels, when a polycistronic vector was used that resulted in higher protein levels of Mef2C and lower levels of Gata4 and Tbx5 (MGT vector) which significantly enhanced reprogramming efficiency compared to separate G, M, T transduction as supported by cardiac specific gene expression such as cTnT. In addition, this MGT vector has resulted in more than a 10-fold increase in the number of mature beating cardiomyocytes. On the other hand, addition of an extra transcription factor Hand2 also enhanced reprogramming efficiency (Song et al. 2012). In addition, combinations of small molecules such as SB431542, CHIR99021, 6-bromoindirubin-3′-oxime (BIO), Lithium chloride (LiCl) to replace transcription factors have also been reported to induce cardiac reprogramming (Cao et al. 2016). Of note, CHIR99021 is a GSK3 inhibitor which can up regulate canonical Wnt signalling, and significantly increased cardiac reprogramming efficiency.
<table>
<thead>
<tr>
<th>Species</th>
<th>Cell types</th>
<th>Reprogramming factors</th>
<th>Delivery Method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Embryonic Fibroblasts</td>
<td>Gata4, Mef2c, Tbx5, Hand2, Nkx2.5</td>
<td>Lentivirus</td>
<td>(Addis et al. 2013)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Embryonic Fibroblasts</td>
<td>Gata4, Mef2c, Tbx5, miR133</td>
<td>Retrovirus/Lentivirus miRNA transfection</td>
<td>(Muraoka et al. 2014)</td>
</tr>
<tr>
<td>Mouse</td>
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<td>Mef2c, Tbx5, Myocd</td>
<td>Lentivirus</td>
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<tr>
<td>Mouse</td>
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<td>Gata4, Tbx5, Mef2c, Myocd, Srf, Mesp1, Smarcd3</td>
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<td>(Christoforou et al. 2013)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Embryonic and Dermal tail tip Fibroblasts</td>
<td>Gata4, Mef2c, Tbx5, Hand2, Nkx2.5, TGFB inhibitor</td>
<td>Lentivirus</td>
<td>(Ifkovits et al. 2014)</td>
</tr>
<tr>
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<td>Oct4, Sox2, Klf4</td>
<td>Retrovirus</td>
<td>(Efe et al. 2011)</td>
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<tr>
<td>Mouse</td>
<td>Neo-natal cardiac Fibroblasts</td>
<td>miR1, miR133, miR208, miR499, JAK inhibitor</td>
<td>Plasmid</td>
<td>(Jayawardena et al. 2012)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Post-natal cardiac and dermal tail tip Fibroblasts</td>
<td>Gata4, Mef2c, Tbx5</td>
<td>Retrovirus</td>
<td>(Ieda et al. 2010)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Adult cardiac and dermal tail tip Fibroblasts</td>
<td>Gata4, Mef2c, Tbx5, Hand2</td>
<td>Retrovirus</td>
<td>(Song et al. 2012)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sca1⁺ side population CSCs</td>
<td>Gata4, Mef2c, Tbx5, Myocd</td>
<td>Lentivirus</td>
<td>(Belian et al. 2015)</td>
</tr>
<tr>
<td>Human</td>
<td>Neo-natal derma, cardiac and ESC</td>
<td>GATA4, MEF2C, TBX5, ESSRG, MESP1</td>
<td>Retrovirus</td>
<td>(Fu et al. 2013)</td>
</tr>
<tr>
<td>Species</td>
<td>Cell Type</td>
<td>Transformed Genes</td>
<td>Vector</td>
<td>Reference</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>Human</td>
<td>Adult dermal and cardiac and Fibroblasts</td>
<td>GATA4, MEF2C, TBX5, HAND2, miR1, miR133</td>
<td>Retrovirus</td>
<td>(Nam et al. 2013)</td>
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<td>Gata4, Tbx5, Baf60c</td>
<td>Lentivirus</td>
<td>(Li et al. 2015)</td>
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<td>(Wystrychowski et al. 2016)</td>
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<tr>
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<td>PEI method</td>
<td>(Park and Takimoto, 2016)</td>
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<td>Human</td>
<td>Fibroblasts</td>
<td>Small molecules</td>
<td>Supplemented with medium</td>
<td>Cao et al. 2016</td>
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<tr>
<td>Mouse</td>
<td>Embryonic fibroblast</td>
<td>MyoD transactivation domain fused Mef2c, Gata4, Tbx5, Hand2</td>
<td>Retrovirus</td>
<td>Hirai et al. 2013</td>
</tr>
<tr>
<td>Mouse</td>
<td>Dermal tail tip, Embryonic and Cardiac fibroblasts</td>
<td>Akt1, Gata4, Mef2c, Tbx5, Hand2</td>
<td>Retrovirus</td>
<td>Zhou et al. 2015</td>
</tr>
<tr>
<td>Mouse</td>
<td>Embryonic fibroblasts</td>
<td>ROCK inhibitor, TGF-β inhibitor, Gata4, Hand2, Mef2c, Tbx5</td>
<td>Retrovirus, and AAV</td>
<td>Zhao et al. 2015</td>
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### Table 1.3. *In vivo* cardiac reprogramming

<table>
<thead>
<tr>
<th>Species</th>
<th>Reprogramming factors</th>
<th>Vector</th>
<th>Delivery Method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Gata4, Mef2c, Tbx5</td>
<td>Retrovirus</td>
<td>Intramyocardial injection</td>
<td>(Qian et al. 2012)</td>
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<tr>
<td>Mouse</td>
<td>Gata4, Mef2c, Tbx5, Hand2</td>
<td>Retrovirus</td>
<td>Intramyocardial injection</td>
<td>(Song et al. 2012)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Gata4, Mef2c, Tbx5, Thymosin β4</td>
<td>Retrovirus</td>
<td>Intramyocardial injection</td>
<td>(Srivastava et al. 2012)</td>
</tr>
<tr>
<td>Mouse</td>
<td>miR1, miR133, miR208, miR499</td>
<td>Lentivirus</td>
<td>Intramyocardial injection</td>
<td>(Jayawardena et al. 2012)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Gata4, Mef2c, Tbx5</td>
<td>Retrovirus</td>
<td>Intramyocardial injection</td>
<td>(Inagawa et al. 2012)</td>
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<tr>
<td>Rat</td>
<td>Gata4, Mef2c, Tbx5, Vegf (121, 165, 189)</td>
<td>Lentivirus/Adenovirus</td>
<td>Intramyocardial injection</td>
<td>(Mathison et al. 2012)</td>
</tr>
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</table>
1.7.2. Delivery of reprogramming factors via lentiviral vectors

Lentiviruses are a subclass of retroviruses with a unique feature allowing them to infect both dividing and non-dividing cells, integrating into the cellular genome. The viral genome is a single stranded RNA which is reverse-transcribed when the virus enters the cell to produce a cDNA copy and then integrated into the cellular genome where it remains as a stable provirus and is passed onto the progeny of the cell when it divides (Shao et al. 2010). As integration of lentiviruses is random, this could potentially disturb the normal genomic arrangement leading to activation of oncogenes. However, it is important to note that, lentiviral vectors have showed a lower possibility to integrate in genome regions that potentially cause cancer than gamma-retroviral vectors (Cattoglio et al. 2007). Another study also showed that, lentiviral vectors did not increase the incidence of tumors or contribute to an earlier onset of tumors in mice compared to retroviral vectors which showed much higher incidence (Montini et al. 2006). A number of biosafety features are incorporated into the design of lentiviral vectors to produce safe gene delivery vectors, this includes inactivation of the 3′LTR, referred to as a self-inactivating vector (Montini et al. 2006). Thus, during the reverse transcription step, the 3′LTR region is copied to 5′ region just upstream of the 5′LTR which can block LTR mediated downstream gene transcription. Therefore, these viral vectors can efficiently infect cells but, following transduction, they are replication-incompetent. Other biosafety features include use of the heterologous glycoprotein of the vesicular stomatitis virus (VSV-G) envelope which confers a broad host range and use of strong CMV or EF1α promoters for transgene expression. Lentiviral based
delivery of the GMT factors has been shown to drive cardiac reprogramming (Chen et al. 2012, Addis et al. 2013), and one study has determined the optimum stoichiometric ratio of Gata4, Mef2c and Tbx5 that is essential for efficient cardiac reprogramming (Wang et al. 2015). In addition, lentiviral based vectors have been used to test the effect of different transcription factor combinations on direct cardiac reprogramming in vitro and (Table 1.2) and in vivo (Table 1.3). Protze and colleagues used a lentiviral based approach to screen optimum transcription factor combinations which can activate a panel of cardiac specific genes to assess the conversion efficiency (Protze et al. 2012). The generation of functional cardiomyocytes was not reported, however, it was demonstrated that the three-factor combination of myocardin (MYOCD), Mef2C and Tbx5 gave a higher expression of cardiomyocyte genes than GMT. Christoforou et al reported that overexpression the transcription factors MYOCD and SRF alone or in combination with Mesp1 and SMARCD can reprogram mouse embryonic fibroblasts (MEFs) into cardiomyocytes, with a greater reprogramming efficiency than has been observed when MYOCD and SRF were used together (Christoforou et al. 2013). Furthermore, Ifkovits and colleagues have demonstrated that lentiviral expression of transcription factors Hand2, Nkx2.5, Gata4, Mef2C, and Tbx5 together with the TGFβ inhibitor, SB431542 (SB) could significantly enhance MEF reprogramming into beating cardiomyocytes. Interestingly, human adipose tissue derived mesenchymal stem cells (ADMSCs) have also been reprogrammed into beating cardiomyocytes with lentiviral overexpression of a combination of the transcription factors Gata4, Tbx5 and Baf60c (Li et al. 2015). Jayawardena et al demonstrated
that *in vivo* reprogramming of mouse cardiac fibroblasts into cardiomyocytes could be achieved by lentiviral delivery of microRNAs (miR-1, miR-133, miR-208 and miR-499) (Jayawardhena et al. 2015).

Reprogramming via retroviral or lentiviral transduction with defined transcription factors is an inefficient process ranging from 0.01% to 0.1% for iPS cell generation and requires a viral transduction efficiency greater than 30% and an average of 15 integrated proviral copies to reprogram MEFs into iPS cells (Wernig et al. 2008). In addition, it was also shown that efficient iPS reprogramming depends on the internal promoter, and reprogramming efficiency was significantly higher when a CMV promoter was used compared to EF1 α (Mali et al. 2008; Zhao et al. 2008).

1.8. **Non-integrating vectors**

1.8.1. **Delivery of reprogramming factors via plasmid vectors**

Transcription factors delivered by integrating viral vectors can become transcriptionally silenced over time through *de novo* DNA methylation, and transgene expression can become spontaneously reactivated during cell culture and differentiation (Aoi et al. 2008; Nakagawa et al. 2008). To avoid potential genome effects due to transgene integration during the reprogramming process non integrating gene transfer methods have been developed. To this end, transient gene expression approaches have been tested in iPS cell reprogramming. Thus, these iPS cells are devoid of integrated transgenes (Okita et al. 2008). In addition, Gonzalez et al. generated iPS cells from MEFs by transient transfection of a single polycistronic vector encoding the four factors, and showed that these iPS cells
were also free of transgene integration (Gonzalez et al. 2009). However, the iPS generation efficiency using a plasmid transfection approach is considerably lower (1–29 colonies from 1,000,000) than that by retroviral and lentiviral methods. Adler et al reported that direct conversion of MEFs into functional neurons using non-viral plasmid vectors (Adler et al. 2012). In this study, plasmids encoding the neuronal transcription factors Brn2, Ascl1, and Myt1l (BAM) were delivered to MEFs, and reported up to 7.6% of neuronal specific Tuj1+ cells could be obtained from BAM transfected cells, which expressed neuron specific MAP2 (microtubule-associated protein 2), tau, and synaptophysin. These reprogrammed cells exhibited neuron like morphology and demonstrated action potentials in response to a depolarizing current injection. In addition, Jayawardena et al reported that plasmid vector based microRNA delivery to murine fibroblasts can promote direct cardiac reprogramming (Table 1.2 and 1.3) (Jayawardena et al., 2012). By screening of pool of miRNAs, they identified a combination of microRNAs (miRNA) 1, 133, 208, and 499 capable of inducing direct conversion of fibroblasts to cardiomyocytes. Transfection of a pool of these miRNAs could repress fibroblastic genes such as vimentin and Ddr, whilst inducing mature cardiomyocyte markers such as cTnT, Mhy6, Mhy7. An αMHC reporter assay revealed that miRNA mediated reprogramming efficiency was 7%. In addition, the reprogrammed cells exhibited sarcomeric organization, and showed spontaneous calcium transients and reprogramming could be further enhanced by treatment with a JAK inhibitor by 10 fold.
1.8.2. Minicircle DNA vectors

To overcome problems associated with conventional plasmid vectors, minicircle DNA vectors were developed (Kay et al. 2010). Minicircle (MC) is defined as extra chromosomal supercoiled plasmid DNA which contains only the mammalian expression cassette but is devoid of the bacterial backbone (Kay et al. 2010). Conventional plasmid vectors contain bacterial DNA sequences such as origin of replication, antibiotic resistant gene, which have been found to be responsible for dramatic silencing of the delivered transgenes (Chen et al. 2004b). The MC is a plasmid-based gene transfer vector system containing only the expression cassette and thus devoid of bacterial sequences (Kay et al. 2010). In principle, a parental plasmid is constructed consisting of the eukaryotic expression cassette flanked by bacterial (attB) and bacteriophage (attP) attachment sites, and this strategy has allowed the placement of sequences needed for plasmid propagation in bacteria outside these attachment sites. Induction of recombination between the attachment sites in bacteria with a recombinase such as phiC31 (Maniar et al. 2013) can produce two MCs containing the gene of interest with suitable regulatory sequences and other MC contained the remaining of plasmid sequences together with multiple copies of restriction sites such as I-SceI (Kay et al. 2010). During the MC induction, the host bacterium has been genetically modified to produce I-SceI restriction endonuclease and thus the second MC is degraded. Since bacterial sequences are removed from minicircle vectors, they have been shown to be superior to conventional plasmid vectors especially exhibiting lower gene silencing and improved transfection efficiencies in vitro (Dad et al. 2014) and in vivo (Dietz
et al. 2013). Using a minicircle vector expressing LIN28A, NANOG, SOX2, OCT4, and a GFP marker in human adipose stromal cells was able to reprogram them into iPS cells (Jia et al. 2010, Narsinh et al. 2011). In a separate study (Monjezi et al. 2017), MC vectors has been used to engineer T cells to produce a chimeric antigen receptor (CAR) which is specific for CD19, and have demonstrated a therapeutic effect of this engineered CD19-CAR T-cell against B-cell malignancies. In their system, they constructed two MC vectors which carry the CAR gene and the pT2 transposon and another MC was engineered to express a transposase. Delivery of both the MCs into T-cells allowed efficient transposition of the CAR gene, and they have demonstrated that injection of these engineered T-cells into a lymphoma disease mouse model completely cleared lymphoma from the mouse bone marrow.

1.8.3. Replicating Episomal plasmids

Regular plasmid vectors lack the ability to replicate in mammalian cells and are lost with cell division. However, oriP/Epstein–Barr nuclear antigen-1 (EBNA1) based plasmid vectors can replicate as an extra chromosomal circular DNA without integration in both dividing and non-dividing cells (Yates et al. 1984, Yates et al. 1985). Thus, these episomal plasmid vectors can increase the expression of transgenes during the reprogramming process and may enhance reprogramming. In this system, oriP elements (cis-acting components) and EBNA1 (trans-acting component) drive the stable extra chromosomal replication of oriP/EBNA1-based episomal vectors. This system has been used to generate iPS cells which are free
from reprogramming factors and residual vector sequences (Yu et al. 2009). oriP/EBNA1 vectors have been used to introduce reprogramming factors into human fibroblasts, and can be subsequently removed from cells by culturing in the absence of drug selection (Yu et al. 2009). oriP/EBNA1 vectors can replicate stably as an extra chromosomal form and requires only a cis-acting oriP element (Yates et al. 1984) and a trans-acting EBNA1 gene (Yates et al. 1985). As it has been reported previously, the oriP/EBNA1 vectors replicate only once per cell cycle and with drug selection they can be established as stable episomes in about 1% of the initial transfected cells (Leight et al. 2001). When the drug selection is removed, the episomes are lost, using this method cells free of plasmids can be obtained after reprogramming (Nanbo et al. 2007).

1.8.4. Delivery of reprogramming factors via adenovirus

Adenoviruses are non-integrating episomal vectors, remaining in an extra chromosomal form. Adenoviruses can efficiently infect all cell types and delivered transgenes can be highly expressed. Adenoviruses delivered transgenes are lost during the cell division and during the reprogramming process. It has been shown that, the reprogramming of iPS cells from mouse liver cells and fibroblast by using non-integrating adenoviruses transiently expressing OCT4, SOX2, KLF4, and c-MYC. They have demonstrated that these iPS cells could express endogenous pluripotency markers SOX2 and SSEA-1, form teratomas and differentiate into representative cell types of the three germ layers including muscle, cartilage, and epithelial cells. However, the reprogramming efficiency of adenoviruses has been
significantly low (0.0006%) compared with retroviruses and lentiviruses (0.01-0.1%) (Stadtfeld et al., 2008).

1.8.5. Integrase defective lentiviral vectors

Integration of reverse transcribed viral cDNA into the cellular genome is mediated by the viral enzyme integrase (IN) (Arhel, 2010). There are three functional domains within the lentiviral IN, which are, a zinc binding N-terminal domain, a Mg\(^{2+}\)-dependent central catalytic core domain, and a C-terminal DNA binding domain (Qamar Saeed et al. 2014). Therefore, it is appreciated that mutation of IN within lentiviral vectors may provide an integration defective form of the vector (IDLV). These IDLVs can still transduce cells efficiently without integrating the delivered transgenes into the cellular genome (Naldini et al. 1996) existing as extra chromosomal double-stranded DNA forms, which is removed with cell division and thus they can achieve short-lived transgene expression (Vargas et al. 2004; Nightingale et al. 2006). There are several classes of mutants in which typical class I mutations are those modifying any of the three amino acids (D64, D116, and E152) of the catalytic domain (Engelman, 1999). Among them, the D64V substitution is the most popular IDLV (Sarkis et al. 2008; Wanisch et al. 2009). The most common class 2 mutations are D167 and the Q168 residues, such as Q168A (Bouyac-Bertoia et al. 2001) and D167H (Qamar Saeed et al. 2014). Yang et al reported that D64V mutation derived IDLV can be used to purify human hepatic progenitors (Yang et al. 2013). In this study they used an IDL vector which expresses GFP under the control of liver-specific apolipoprotein A-II (APOA-II)
promoter and this was used to transduce human ESC, following differentiation into hepatic progenitors, they were sorted and these APOA-II-GFP-positive cells expressed hepatoblast markers such as α-fetoprotein and cytokeratin 19. Interestingly, Lau et al demonstrated that over expression of Ascl1, Brn2 and Myt1L in human fibroblast using IDLV (D64V mutation derived IDLV) could reprogram fibroblast into functional neuron in vitro (Lau et al. 2014).

1.9. Excisable integrating vectors

1.9.1. Delivery of reprogramming factors via viral vectors with floxed transgenes

Excisable integrating vectors are attractive for cellular reprogramming applications and a recently developed lentiviral vector has been described that contains a DOX-inducible minimal CMV promoter for driving transgene expression and a loxP site located in the 3′ LTR (Soldner et al. 2009). Similar to the SIN lentiviral vector technology, upon reverse transcription, the loxP site is duplicated into the 5′ LTR resulting in genome integration of the transcription factor flanked with loxP sites. Therefore, these reprogramming factors (floxed factors) can be excised upon Cre-recombinase expression in the reprogrammed iPS cells (Soldner et al. 2009). Furthermore, it is noteworthy that, when each of the floxed reprogramming factors (iPS factors) were delivered separately and independently integrated into different sites within the genome, multiple excisions of the transgenes could be performed by Cre recombinase, which could potentially lead to genome rearrangement and
genomic instability. A combination of polycistronic technology with loxP flanked transgenes may be a safer approach.

It has been reported that cloning of a single polycistron encoding the 2A sequences linked to iPS reprogramming factors in a self-inactivating (SIN) lentiviral vector with a loxP site in the truncated 3’ LTR. The polycistron was flanked by the two loxP sites in the 5′ and 3′ LTRs, (Chang et al. 2009), with expression of Cre recombinase in these iPS cells resulting in excision of the integrated transgenes.

1.9.2. Delivery of reprogramming factors via PiggyBac transposon/transposase system

Transposons are mobile genetic elements also referred to as jumping genes moving from one genomic site to another site through a process called transposition which can also cause genomic mutations. Transposons can be grouped into two classes. Class I mobile genetic elements can make copies which are integrated into the cellular genome. Then, the DNA is transcribed to RNA, and subsequently reverse transcribed to cDNA which is inserted into another genetic locus (Mc Clintock et al. 1950). Class II mobile genetic elements move directly from one site to another using a transposase for excision from their original locus and insertion into a new locus within the genome.

The PiggyBac (PB) transposon belongs to the class II mobile genetic elements and requires only a 13 bp inverted terminal repeats (ITR) and an active transposase enzyme (594 amino acids) to either insert or excise a transgene (Cary et al. 1989;
Fraser et al. 1996). The PB based vector has been used for iPS cell generation from various species such as murine fibroblasts (Woltjen et al. 2009), human fibroblasts (Kaji et al. 2009), and equine fibroblast (Nagy et al. 2011). It has been shown that efficient elimination of the transcription factors from reprogrammed cells upon transposase expression (Woltjen et al. 2009; Kaji et al. 2009) is possible. PB systems have several advantages when compared to other gene delivery systems such as,

1. PB transposition is technically simpler than other gene-delivery systems. Compared to the common viral vectors, only a simple plasmid preparation is required.
2. A broad range of cells are amenable for PB transposition.
3. Transgenes and PB transposon insertions can be removed from the genome. Therefore, it provides lower genomic modifications in reprogrammed cells (Kaji et al. 2009; Woltjen et al. 2009)

However, similar to plasmid vectors, PB exhibit lower gene transfer efficiency compared to viral vectors.

1.10. Viral RNA-based reprogramming using Sendai virus (SeV)

SeV-based vectors have been used to induce reprogramming with iPS factor expression which are delivered as RNA and undergo direct translation in the cytoplasm (Li et al. 2000). SeV is an enveloped virus and a member of the family Paramyxoviridae with a non-segmented negative-strand RNA genome (Nagai et al. 2004), which contains 15,384 nucleotides and includes six cistrons (Shioda et al.
1986). SeV vectors rely only on the virus-encoded RNA polymerase and ubiquitous cellular tubulin for their gene expression (Garcin et al. 1995). SeV replication is independent of cellular nuclear factors and does not involve reverse transcription or genomic integration undergoing a DNA phase.

Accumulating evidence has demonstrated that recombinant SeV vectors can be used to induce strong exogenous gene expression in the cardiovascular system (Masaki et al. 2001), rat retinal epithelium (Murakami et al. 2008), rat parenchymal hepatocytes (Fujita et al. 2008), rat cortical neurons (Li et al. 2000). Furthermore, Shibata and colleagues have reported that IFN-beta gene transfer into murine dendritic cells could enhance antitumor immunity in mice (Shibata et al. 2006). In the context of cellular reprogramming, SeV mediated delivery of iPS factors (OSKM) has been shown, in which mouse fibroblasts could be reprogrammed into iPS cells with approximately 1% reprogramming efficiency (Nishimura et al. 2011). Interestingly, intramuscular delivery of SeV mediated FGF2 and VEGF into mice with critical limb ischemia has been shown to increase angiogenesis (Masaki et al. 2002; Onimaru et al. 2002).

1.11. Control of cellular signalling pathways for efficient reprogramming

Previous studies have revealed that the TGF-β pathway plays a critical role in profibrotic gene expression (Tomasek et al. 2002; Davis et al. 2014). Zhao et al demonstrated that, targeted inhibition of TGF-β signalling with A83-01, a selective inhibitor of TGF-β signalling (Tojo et al. 2005) could significantly decrease
phosphorylation of Smad2, and inhibited expression of fibroblastic genes Fn-EDA, Col1a1 and Col3a1, whilst increased cardiac specific genes cTnT and alpha actinin expression (Zhao et al. 2015).

In addition to the TGF-β pathway, ROCK signalling is also a repressor of cardiac reprogramming of fibroblasts. Rho stimulates pro-fibrotic events via activation of its downstream effector ROCK (Zhao et al. 2015), and thus addition of a ROCK inhibitor Y-27632 to fibroblasts decreased expression of Fn-EDA and αSMA and significantly increased cardiomyocyte specific gene expression, cTnT and alpha actinin, together with enhanced beating cardiomyocytes clusters (Zhao et al. 2015). In contrast to the TGF-β pathway, Wnt signalling has also been shown to promote cardiac differentiation of ESCs (Davidson et al. 2012). Interestingly, Cao et al and Fu et al separately demonstrated that activation of Wnt signalling by inhibition of GSK3 through the small molecule CHIR99021 could significantly enhance fibroblast conversion into cardiomyocytes (Cao et al. 2016, Fu et al. 2015). Similar to Wnt signalling, the Akt pathway has also been shown to promote cardiac differentiation in which, Olsen and colleagues reported that the addition of Akt1 protein to GMT transcription factors could markedly enhance mouse tail tip and cardiac fibroblast conversion into cardiomyocytes (Zhou et al. 2015). These approaches will improve reprogramming and cardiac regeneration.
1.12. Nitric oxide

Nitric oxide (NO) is a diatomic free radical gas and one of the most important signalling molecules in mammalian physiology. NO mediates variety of physiological events such as smooth muscle relaxation, vasodilatation, neurotransmission, inhibition of platelet aggregation, immunomodulation (Beckman et al. 1996; Bryan et al. 2009; Murad, 2006), and bone remodeling (Taylor et al. 2006; Wang et al. 2009). At the cellular level, NO regulates cell growth, survival, apoptosis, proliferation, and differentiation (Ciani et al. 2004; Mora-Castilla et al. 2010). In addition, NO plays a role in the pathophysiology of several diseases such as diabetes, cancer, cardiovascular disease, and various inflammatory conditions, either through reduced bioavailability or overproduction of NO (Ying and Hofseth 2007, Abramson, 2008; Bryan et al. 2009). NO is synthesized by nitric oxide synthases (NOSs) through oxidation of L-arginine into Nω-hydroxy-L-arginine which produces L-citrulline and NO (Murad, 2006). There are three isoforms of NOS named NOS I/1 (neuronal, [nNOS]), NOS II/2 (inducible [iNOS]) and NOS III/3 (endothelial NOS [eNOS]) (Bryan et al. 2009; Murad, 2006), which utilize a complicated array of cofactors and co-substrates such as molecular oxygen, NADPH, tetrahydrobiopterin, flavin mononucleotide, flavin adenine dinucleotide, heme, and calcium/calmodulin for the synthesis of NO (Bryan et al. 2009, Murad, 2006)
1.12.1. Nitric oxide synthase

Nitric oxide synthases are usually referred to as `dimeric' in their active form without considering calmodulin (CaMs) association (Masters et al. 1996; Hemmens and Mayer, 1997). Thus, in the activation state they exist as tetramers since two NOS monomers can associate with two CaMs (Lee et al. 1995; Hellermann and Solomonson, 1997; Venema et al. 1997). In addition, several co-factors are also tightly bound with NOS during its activation, such as (6R)-5, 6, 7, 8-tetrahydrobiopterin (BH4), FAD, FMN and iron protoporphyrin IX (haem) (Klatt et al. 1995; Stuehr, 1997; Hellermann and Solomonson, 1997; List et al. 1997). Activated NOS can catalyze the conversion of L-arginine, NADPH, and oxygen to the free radical NO, and by products citrulline and NADP (Knowles and Moncada, 1994; Marletta, 1994; Nathan and Xie, 1994).

The three different human NOS isoforms have a 51-57% sequence homology (Liu and Huang, 2008; Alderton et al. 2001). nNOS is commonly found in neuronal tissue, iNOS is found in a wide range of cells and tissues and eNOS is the major isoform found in vascular endothelial cells (Cirino et al. 2003; Wilcox et al. 1997). The expression patterns of these isoforms are different as both eNOS and nNOS are expressed constitutively while iNOS expression is inducible (Alderton et al. 2001) and eNOS and nNOS expression are calcium dependent whilst iNOS is calcium independent.
1.12.2. NOS structure

All of the NOS isoforms exist as a single copy gene in the human genome (Nakane et al. 1993; Hall et al. 1994; Geller et al. 1993; Sherman et al. 1993; Charles et al. 1993) (Table 1.4), and have a similar genomic structure, suggesting evolution from a common ancestral NOS gene (Alderton et al. 2001). As shown in figure 1.1, structurally, NOS contains two domains, an N-terminal oxygenase domain containing binding sites for haem, BH4 and L-arginine. The N-terminal domain is linked by a CaM-recognition which resides at the C-terminal reductase domain which contains binding sites for FAD, FMN and NADPH (McMillan et al. 1995; Richards et al. 1994; Ghosh et al. 1995). The crystal structures have been solved for truncated oxygenase domains of murine iNOS (Crane et al. 1997; Crane et al. 1998), human iNOS (Fischmann et al. 1999; Li et al. 1999), bovine and human eNOS (Raman et al. 1998) with a variety of ligands bound to the structure. To date, the nNOS oxygenase domain structure has not been solved.
<table>
<thead>
<tr>
<th>Human NOS isoform</th>
<th>Gene structure and size</th>
<th>Chromosomal location</th>
<th>Number of amino acids (aa), protein size</th>
<th>GenBank Accession numbers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOS</td>
<td>29 exons, 28 introns, 200 kbp</td>
<td>12q24.2±12q24.3 of chromosome 12</td>
<td>1434 aa, 161 kDa</td>
<td>L02881, U11422</td>
<td>(Nakane et al. 1993; Hall et al., 1994)</td>
</tr>
<tr>
<td>iNOS</td>
<td>26 exons, 25 introns, 37 kbp</td>
<td>17cen±q11.2 of chromosome 17</td>
<td>1153 aa, 131 kDa</td>
<td>LO9210, L24553, X73029</td>
<td>(Geller et al. 1993; Sherman et al. 1993; Charles et al. 1993)</td>
</tr>
<tr>
<td>eNOS</td>
<td>26 exons, 25 introns, 21±22 kbp</td>
<td>7q35±7q36 of chromosome 7</td>
<td>1203 aa, 133 kDa</td>
<td>M93718, M95296</td>
<td>(Janssens et al. 1992; Marsden et al. 1992)</td>
</tr>
</tbody>
</table>
Figure 1.1. Domain structure of human nNOS, eNOS and iNOS. Oxygenase, reductase and PDZ domains are illustrated by solid boxes. Myristoylation (Myr) and palmitoylation (Palm) sites on eNOS are shown as solid green and blue lines respectively in the oxygenase domain. The autoinhibitory loop within the FMN regions of nNOS and eNOS is also indicated.

1.12.3. Regulation of NOS

NOSs are regulated by a variety of cellular mechanisms. Cellular and tissue specific localization of the NOS isoforms can be regulated by transcriptional and translational regulation (Geller et al. 1998; Forstermann et al. 1998). Table 1.5 summarizes the regulation of the NOS activity through expression of the NOS isoforms by protein-protein interactions, alternative mRNA splicing and covalent modification.

Tetrahydrobiopterin (BH₄) is an important cofactor for NOS since it couples with heme iron to synthesis NO, and is needed for iNOS to dimerize in animal cells (Stuehr, 1997). It is also reported that binding of the first BH₄ to NOS lowers the enzyme's affinity for binding the second BH₄ by at least an order of magnitude (Gorren et al. 1996), and suggested that NOS may contain only one BH₄ per
dimer. In this scenario, NOS bound to a BH₄ subunit may generate NO while unbound NOS may generate superoxide or H₂O₂ (Gorren et al. 1996), and this may specific to the nNOS, which may produce high levels of H₂O₂ in the absence of BH₄ (Gorren et al. 1996; Mayer et al. 1997). It has been shown that when cells were induced to express iNOS, genes encoding BH₄ biosynthetic enzymes are also activated, providing increased BH₄ levels for dimer assembly and coupled NO synthesis (Mayer and Werner, 1995). On the other hand, insufficient BH₄ can limit NO synthesis in cells expressing nNOS or eNOS as documented previously (Harrison, 1997; Cosentino et al. 1998).

Regulation of NOS activity through alternative mRNA splicing has been reported with nNOS (Brenman et al. 1997) and iNOS (Eissa et al. 1996) but interestingly not with eNOS. Six different molecular species of nNOS mRNAs have been shown to be expressed in a developmentally regulated manner, and therefore, alternative splicing regulates the synthesis of different nNOS in terms of both enzymatic characteristics and structural features (Brenman et al. 1997). In this investigation, they reported that, one alternatively spliced nNOS isoform which was not contained with exon 2, showed full enzymatic activity but lacked a major protein-protein interaction domain (PDZ domain) responsible for targeting nNOS to synaptic membranes, revealing that alternative splicing produces a mislocalised but fully active protein.
Table 1.5. Regulation of NOS

<table>
<thead>
<tr>
<th>Type of Regulation</th>
<th>nNOS</th>
<th>eNOS</th>
<th>iNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternate mRNA splicing</td>
<td>Yes</td>
<td>Not reported</td>
<td>Yes</td>
</tr>
<tr>
<td>Protein-protein interactions</td>
<td>CaM/Ca(^{2+}), PDZ domains, Caveolin-1, Hsp90</td>
<td>CaM/Ca(^{2+}), Caveolin-1, Caveolin-3, Hsp90, ENAP-1</td>
<td>CaM/Ca(^{2+}), kalirin</td>
</tr>
<tr>
<td>Covalent modifications</td>
<td>Phosphorylation</td>
<td>Myristoylation, palmitoylation, phosphorylation</td>
<td>Not yet reported</td>
</tr>
</tbody>
</table>

ENAP-1: eNOS-associated protein-1; CaM: Calmodulin; Hsp90: Heat-shock protein 90; PDZ: PSD-95 (post synaptic density protein 95) discs large/ZO-1 homology; PIN: Protein inhibitor of NOS.

1.12.4. Regulation of eNOS activity

1.12.4.1. Calmodulin (CaM)

CaM was the first protein shown to interact with NOS (Bredt and Snyder, 1990) and is necessary for its enzymatic activity. eNOS requires Ca\(^{2+}\) availability for NO synthesis. CaM binding increases the rate of electron transfer from NADPH to the reductase domain flavins and artificial electron acceptors such as ferricyanide and cytochrome c (Gachhui et al. 1996; Gachhui et al. 1998). It also triggers electron transfer from the reductase domain to the haem center (Gachhui et al. 1998). eNOS contains 40-50 amino acid inserts in the middle of the FMN-binding subdomain, which has been described as an auto inhibitory loop (Abu-Soud et al. 1994). Analysis of deletion mutants of eNOS within this loop have shown that the insert acts by destabilizing CaM binding at low Ca\(^{2+}\) and by inhibiting electron transfer.
transfer from FMN to the haem in the absence of Ca\textsuperscript{2+}/CaM (Salerno et al. 1997; Nishida et al. 1999).

1.12.4.2. Phosphorylation

Phosphorylation of eNOS can increase NOS activity independent of calcium availability (Corson et al. 1996; Fisslthaler et al. 2000). The protein tyrosine phosphatase inhibitor phenylarsine oxide has been shown to activate eNOS in the presence and absence of extracellular calcium and the calmodulin inhibitor calmidazolium, suggesting that tyrosine phosphorylation is essential for calcium-independent eNOS activation (Fleming et al. 1998). Interestingly, it has been reported that tyrosine phosphorylation is highly dependent on cell passage suggesting that intracellular mechanisms that regulate tyrosine phosphorylation of eNOS can disappear when cells are cultured for prolonged periods (Fleming et al. 1998; Garcia-Cardena et al. 1996).

eNOS can be phosphorylated on serine residues when endothelial cells are exposed to shear stress and to calcium-mobilizing agents (Corson et al. 1996; Michel et al. 1993), and this is governed by protein kinase C (PKC) and cAMP-dependent kinase (protein kinase A [PKA]) (Chen et al. 1999). Michell and colleagues have demonstrated that PKA drives eNOS phosphorylation at Threonine-495 and Serine-1177 of human eNOS in calcium and calmodulin independent manner (Michell et al. 1999) thereby activating eNOS. In addition to PKA and PKC, Akt (protein kinase B [PKB]) can phosphorylate eNOS at Serine-1179 (Fulton et al. 1999; Gallis et al. 1999). McCabe and colleagues have reported
that, Akt driven eNOS phosphorylation reduces the dissociation of calmodulin from activated eNOS (McCabe et al. 2000). Of note, treatment of endothelial cells with an NO donor has been shown to induce serine phosphorylation of eNOS, while inhibiting its activity (Michel et al. 1993), suggesting that excess levels of NO may act as a negative feedback mechanism for eNOS activity, and therefore suggested that, PKC might be the kinase involved in this inhibitory phosphorylation event since PKC driven eNOS phosphorylation is known to inhibit eNOS activity (Hirata et al. 1995).

1.12.4.3. Heat-shock protein 90 (Hsp90)
The molecular chaperone Hsp90 has been identified as a regulator of eNOS activity, possibly as an allosteric modulator (Garcia-Cardena et al. 1998). Activation by vascular endothelial growth factor (VEGF), histamine or fluid shear stress in human endothelial cells increases the interaction between eNOS and Hsp90. This interaction increases eNOS activity by approximately three-fold (Garcia-Cardena et al. 1998). The activity of purified eNOS was also increased by purified Hsp90, suggesting a direct interaction. eNOS associated protein-1 (ENAP-1) was previously identified as an eNOS interaction protein. It is a 90 kDa protein that is tyrosine phosphorylated in response to bradykinin stimulation of eNOS activity in bovine endothelial cells (Venema et al. 1996). Hsp90 is also a 90kDa protein. It is possible that ENAP-1 might be Hsp90. However, it remains to be demonstrated (Venema et al. 1996).
1.12.5. Regulation of eNOS localisation

1.12.5.1. Myristoylation, palmitoylation

In contrast to other NOS isoforms eNOS contains a myristoyl group and it is covalently attached to the glycine residue at the amino terminal domain, thus eNOS can be myristoylated at the N-terminus (Liu et al. 1995; Michel et al. 1999). Myristoylation of eNOS stimulates membrane binding (Liu et al. 1995), and it has been shown that, myristoylation is necessary for membrane localization and activity of eNOS (Sakoda et al. 1995), and when this modification is absent, eNOS is localized to cytoplasm (Liu et al. 1997; Robinson and Michel, 1995). Sessa et al have reported that, myristoylation directs eNOS to the Golgi complex, where it is palmitoylated (Sessa et al. 1995).

Palmitoylation occurs post-translationally and reversibly at cysteine residues Cys$^{15}$ & and Cys$^{26}$ (Liu et al. 1995; Liu et al. 1997). eNOS can be palmitoylated on two cysteine residues (cysteine-15 and -26) at the N-terminus, and this modification is reversible. Prior to palmitoylation, it requires eNOS myristoylation, and stabilizes the association of eNOS with the membrane (Liu et al. 1995). The unique (Gly-Leu)$_5$ repeat between the two palmitoylation sites of eNOS is required for palmitoylation (Liu et al. 1997) and interestingly, it has been documented that, when palmitoylation sites are mutated, eNOS enzyme activity is markedly decreased (Shaul et al. 1996). Garcia-Cardena et al have demonstrated that a mutated palmitoyl-deficient enzyme has an altered cellular localisation compared with wild-type eNOS, resulting in low levels of protein at the plasma membrane (Garcia-Cardena et al. 1996). In addition, NO production was also been impaired
by the palmitoylation-deficient enzyme within endothelial cells (Liu et al. 1996). Therefore, these dual acylations of eNOS are required for efficient localisation to the plasma membrane caveolae of endothelial cells (Shaul et al. 1996).

1.12.6. eNOS localisation in caveolae
Studies have demonstrated that the localisation of eNOS within the cell determines its activity. Caveolae are defined as 60–80 nm invaginations of the plasma membrane that contain oligomeric caveolins (Parton and Simons 2007). It is estimated that each caveola is composed of 140–150 caveolin-1 (CAV-1) proteins (Pelkmans and Zerial, 2005).

1.13. Caveolins in caveolae formation
The membrane protein family of caveolins is the major drivers of caveolae formation (Rothberg et al. 1992; Kurzchalia et al. 1992; Scherer et al. 1996; Way and Parton, 1995). There are three caveolins, CAV-1, CAV-2 and CAV-3. CAV-1 (Figure 2) and CAV-2 are expressed in most cells types excluding skeletal muscle cells, whereas CAV-3 is expressed in striated muscle cells (Parton and del Pozo, 2013). Smooth muscle cells and cardiomyocytes express CAV-1, CAV-2 and CAV-3 (Robenek et al. 2008; Head et al. 2006; Patel et al. 2007). The expression levels of caveolins significantly differ from tissue to tissue. Adipocytes show higher expression levels of caveolin than hepatocytes in vivo (Parton and del Pozo, 2013). However, lymphocytes, neurons and hepatocytes express low but
functionally important levels of caveolin (Tomassian et al. 2011; Fernandez-Rojo et al. 2012; Head et al. 2011). CAV-1 and CAV-3 are essential for the formation of caveolae (Le Lay and Kurzchalia, et al. 2005). Deletion of CAV-1 causes loss of caveolae in non-muscle tissues (Drab et al. 2001; Razani et al. 2002). On the other hand, ectopic expression of CAV-1 in cells lacking endogenous caveolins or caveolae leads de novo formation of caveolae (Fra et al. 1995).

1.13.1. Caveolin-1

Caveolin-1 is a 22-kDa integral membrane protein with both the N-terminus and C-terminus exposed to the cytoplasm (Aoki et al. 2010; Monier et al. 1995). Caveolin-1 is comprises of 178 amino acid residues. The most prominent domain within caveolin-1 is the caveolin scaffolding domain (CSD, residues D82–R101) (Figure 1.2), which has been implicated in membrane attachment (Schlegel et al. 1999), interactions with other proteins (Couet et al. 1997; Liu et al. 2002), including eNOS (Bernatchez et al 2005) and cholesterol binding (Ikonen et al. 2004; Yang et al. 2014).

It has been demonstrated that the localisation of eNOS to caveolae inhibits enzyme activity, and this is governed by caveolin-1 (Ju et al. 1997, Bernatchez et al. 2011). In addition to the caveolae, caveolin-1 (CAV-1) is also resident in the Golgi complex and is known to bind to the caveolae-enriched lipids cholesterol and glycosphingolipids (Conrad et al. 1995). Caveolin-1 interacts with eNOS via its scaffolding domain (amino acid residues 82–101) (Ju et al. 1997; Michel et al.
Garcia-Cardena et al. have demonstrated that eNOS and CAV-1 binding requires both myristoylation and palmitoylation of eNOS (Garcia-Cardena et al. 1997). This interaction can block eNOS binding with CaM which results in reduced eNOS activity (Michel et al. 1997, Ju et al. 1997). In addition to eNOS, other signalling proteins such as phosphatidylinositol 3-kinase, c-Src, and Ha-Ras have also been shown to interact with the CAV-1 scaffolding domain (Li et al. 1996; Couet et al. 1997).

The sequence in human eNOS that mediates the interaction with caveolin-1 has been identified as \(^{350}\text{FSAAPFSGW}^{358}\), by analyzing the scaffolding domain-binding consensus sequences \(\phi X \phi XXX \phi\) and \(\phi XXXX \phi XX \phi\) (\(\phi\) represents Trp, Phe, or Tyr and \(X\) represents any amino acid) (Couet et al. 1997; Garcia-Cardena et al. 1997). The CAV-1 binding motifs of eNOS is located on the oxygenase domain (Ghosh et al. 1998), and this interaction facilitates eNOS to interact with the bradykinin receptor and with the cationic amino acid transporter 1 (CAT-1) (Ju et al. 1998; McDonald et al. 1997). CAT-1 has been shown to participate in the transfer of the \(L\)-arginine (McDonald et al. 1997). In addition, Fujimoto (1993) has demonstrated that a calcium pump which is important for eNOS binding with CaM is also present in caveolae, indicating that most of the components necessary for eNOS activation are located within the caveolae. In order for eNOS activation, within the caveolae, eNOS associates with bradykinin (Prabhakar et al. 1998), calcium ionophore and calcium-bound CaM (Feron et al. 1998), replacing CAV-1 and this results in eNOS being released from the plasma membrane and locating in the cytoplasm (Mitchel et al. 1993). Within the cytoplasm, the activated eNOS-
calmodulin complex generates NO until the intracellular calcium concentration is reduced to the level that CaM dissociates from eNOS and the inhibitory eNOS-CAV-1 complex can be generated indicating an NO mediated negative feedback mechanism (Feron et al. 1998). On the other hand, alanine scanning of the CAV-1 scaffolding region has shown that the threonine residues 90 and 91 (T90, T91) and phenylalanine 92 (F92), are the residues responsible for eNOS inhibition upon CAV-1 binding (Bernatchez et al. 2005). Furthermore, this study revealed that eNOS inhibition disappeared upon overexpression of eNOS with a mutated CAV-1 in which phenylalanine at the amino acid position 92 was replaced with alanine (F92A) (Bernatchez et al. 2005). Interestingly, it has been shown that, delivery of CAV-1 F92A peptides into normal mouse could increase NO levels and lower the blood pressure (Bernatchez et al. 2011). Furthermore, the same group has recently reported that development of mouse model for expression of CAV-1 F92A in an endothelial-specific manner demonstrated NO bioavailability was higher in transgenic mice and blood pressure was also low (Kraehling et al. 2016). Finally, Xia et al have demonstrated that in vitro modification of rat MSCs to co-expresses eNOS and CAV-1 F92A can promote in vitro angiogenesis (Xia et al. 2016).
Figure 1.2. Domain structure of human caveolin-1. Oligomerization (black and blue), scaffolding (blue) and intramembrane (gray) domains are illustrated. The amino acid sequence of the caveolin-1 scaffolding domain (CSD) is shown.

1.14. Nitric oxide and stem cell differentiation

NO drives stem cell differentiations through cyclic GMP (cGMP) dependent mechanisms, epigenetic modifications (Hickok et al. 2013; Spallotta et al. 2010) and modulating other cellular signalling pathways such as Notch (Charles et al., 2010), Wnt (Du et al. 2013; Bandara et al. 2016). The cGMP dependent mechanism is mediated through its receptor soluble guanylyl cyclase (sGC). cGMP independent effects of NO can also be mediated through its interaction with metal complexes, oxygen (O₂) and superoxide (O₂⁻) that mediate various downstream events. Interaction of NO with oxygen species leads to nitrosylation and nitration of proteins. These posttranslational modifications particularly occur during an
inflammatory response and in cases where superoxide dismutase is not able to scavenge excess superoxide (O$_2^-$) (Beckman et al. 1996).

The significant role of the NO in regulation of stem differentiation has been demonstrated (Krumenacker et al. 2006; Mujoo et al. 2006; Sharin et al. 2011), where alteration of intracellular NO levels can modulate the differentiation of bone marrow derived progenitor cells (Michurina et al. 2004; Guthrie et al. 2005). Exposure of CD34+ bone marrow derived cells to NO donors showed inhibition of erythroid but induction of myeloid colony formation (Shami and Weinberg, 1996). Furthermore, NO production by murine bone marrow cells enhanced the colony formation ability of isolated Sca-1 and Thy-1 positive cells (Bøyum et al. 2004). On the other hand, NO can also regulate hematopoietic stem cell number and function in vivo. This was demonstrated by exposure of mice to NOS inhibitors and showing an increase in the number of hematopoietic stem cells compared to the untreated mice (Michurina et al. 2004). eNOS knockout mice have demonstrated impaired mobilization of bone marrow stem and progenitor cells (Aicher et al. 2003) which may be related to impaired neo-vascularization. Moreover, eNOS knockout mice exhibited reduced endothelial progenitor cell development from hematopoietic stem cells and were unable to re-vascularize (Guthrie et al. 2005). The role of NO in heart development has also been demonstrated with eNOS being detected during the early stages of cardiomyogenesis in mouse embryos (Bloch et al. 1999; Mujoo et al. 2008), and increased expression of eNOS during ES cell differentiation. On the other hand, treatment of these cultures with the NOS
inhibitor L-NAME inhibited cardiomyocyte differentiation (Bloch et al. 1999). It has previously been demonstrated that NO can promote ES cell-derived cardiomyogenesis through exogenous treatment with NO donors or by overexpression of the NOS2 gene (Kanno et al. 2004). In relation to bone development, it has been shown that an eNOS knockout mouse exhibited reduced bone mass (Aguirre et al. 2001), and exogenous treatment with NO donors promoted osteogenic differentiation of ES cells (Ehnes et al. 2015). On the other hand, studies have revealed that Wnt signalling can regulate cardiac differentiation of stem cells (Lian et al. 2012). However, still there is no evidence to show how NO regulates cardiac differentiation of stem cells through regulation of Wnt signalling.

NO can regulate the interaction of transcription factors with chromatin by modulating histone acetylation events (Huang and Reichardt, 2001, Nott et al. 2008). It has been revealed that during transcriptional activation, the tightly compacted chromatin is modified to be in an open chromatin state by histone acetyltransferases at gene promoters, and this modification can be inhibited by histone deacetylases (HDACs) (Cheung et al. 2000; Berger, 2007). Interestingly, it has been demonstrated that NO could drive nitrosylation on HDAC2 in neuronal precursor cells and which induced its release from chromatin and which subsequently could increase Acetylation of histones neighboring neuronal specific neurotrophin-dependent gene promoters such as brain-derived neurotrophic factor (BDNF) promoter, which resulted in the activation of BDNF promoting neuronal development (Nott et al. 2008).
Similar to acetylation, methylation is another epigenetic modification event mediated by NO on lysine (Lys) residues (Hickok et al. 2013). Hickok et al have demonstrated that NO can inhibit the activity of demethylase KDM3A by forming a nitrosyliron complex and thus promoting methylation on Lys9 on histone 3 by recruiting two methyl groups (H3K9me2) (Hickok et al. 2013). Similar to acetylation, methylation of histones also promotes open chromatin conformation, and an increase in surrounding gene transcription.

1.15. The Wnt signalling pathways

Wnt proteins have been identified as cysteine-rich secreted glycoproteins which have been known to regulate development, cell proliferation, cell differentiation, (Cadigan and Nusse, 1997, Huelsken and Birchmeier, 2001 and Lee et al. 2006). Wnts bind to receptors belonging to the Frizzled (FZD) family residing on cell membranes to initiate distinct signal transductions classified as either canonical or non-canonical. There are 19 Wnt proteins which have been identified in mammals (Wodarz and Nusse, 1998). Wnt1, 2, 3 and 3a, 8 and 8b have been identified as the canonical Wnts, whilst Wnt4, 5a, 5b, 6, 7a and 11 have been identified as non-canonical (Huelsken and Behrens, 2002).
1.15.1. Canonical Wnt signalling pathway (β-catenin dependent pathway)

β-catenin is the central molecule of canonical Wnt signalling and in the absence of Wnts binding to FZD receptors, β-catenin is associated with APC (adenomatous polyposis coli) and Axin. This association can promote the phosphorylation of β-catenin by casein kinase 1α (CKIα) and glycogen synthase kinase 3β (GSK3β) leading subsequent degradation of β-catenin by the β-TrCP mediated ubiquitin/proteasome pathway (Liu et al. 2002). When Wnts interact with FZD and LRP5/6 receptors, Disheveled (Dvl) can be hyperphosphorylated by CKIα, promoting FZD and FRAT association resulting in the assembly of an LRP5/6–Axin–FRAT complex that leads to axin degradation (Tolwinski et al. 2003; Klein et al. 2006), and subsequently results in β-catenin being released from the APC–Axin–GSK3β complex and translocated to the nucleus. In the nucleus, β-catenin can replace transcription inhibitors such histone deacetylases (HDACs) from the T cell-specific factor/lymphoid enhancer-binding factor (TCF/LEF) and recruit histone acetylase CBP/p300 to activate downstream gene transcription (Daniels and Weis, 2005) and has been shown to regulate lineage specific genes transcription such as endothelial specific Kdr 1 (Hübner et al. 2017), cardiac specific Gata 4 (Davidson et al. 2012) and osteoblast specific Runx2 (Gaur et al. 2005).

1.15.2. Non-canonical Wnt signalling pathway (β-catenin independent pathway)

Non-canonical Wnt signalling has been regarded as being β-catenin independent and has been less characterized (Ishitani et al. 2003). The best-characterized β-catenin-independent pathway is the planar cell polarity (PCP) pathway (Liu et al.
The PCP pathway is initiated by the activation of FZD and co-receptors, such as Ror2 and Ryk which can activate a cascade which contains small GTPases, RAC1 and Ras homology gene family, member A (RHOA), and c-Jun N-terminal kinase (JNK) as downstream effectors which can control cytoskeleton rearrangements and gene expression (Simons and Mlodzik, 2008).

Figure 1.3. Schematic of the Wnt signalling pathways. Canonical Wnt pathway is activated through β-catenin when canonical Wnt ligands are associated with FZD receptors and LRP5/6 co-receptors. β-catenin independent non-canonical Wnt signals can be transduced through FZD receptors and Ror2 co-receptors to the Dvl-dependent or Ca²⁺ dependent signalling pathways. (Ling et al. 2009)
1.16. Wnt signalling in somatic stem cells

The Wnt signaling pathway plays a pivotal role in the specification and maintenance of stem cell lineages (Boras-Granic et al. 2008; Reya et al. 2003; Zechner et al. 2003). In 1998, Clevers and co-workers reported that elimination of the β-catenin interaction partner TCF4 in mice resulted in complete removal of the stem cell population in the small intestine (Korinek et al. 1998). Furthermore, it was shown that canonical Wnt signalling cooperates with BMP and Notch signaling in the intestinal stem cell niche to control stem cell self-renewal (Clarke, 2006). In the hematopoietic system, Wnt3a can promote self-renewal and proliferation of hematopoietic stem cells (Reya et al. 2003; Willert et al. 2003). On the other hand, it has been shown that regulation of hematopoietic stem/progenitor lineage commitment during hematopoiesis is regulated by Wnt (Nemeth et al. 2009; Malhotra et al. 2009). Canonical Wnt signalling is also crucial for heart development (Dravid et al. 2005; Naito et al. 2006). Interestingly, previous studies have shown that canonical Wnt signalling can promote cardiac differentiation of ES cells (Lian et al. 2012). Furthermore, overexpression of β-catenin inhibitors such as Axin (Hsu et al. 2001) or loss of LEF1 function inhibits mammary gland differentiation from precursor cells (Boras-Granic et al. 2006). Other studies have revealed the role of Wnt signalling in neoangiogenesis where nuclear localization of β-catenin increased angiogenesis associated gene transcription and subsequently promoted vessel formation (Shivanna et al. 2015). Wnt signalling has been shown to be associated with myogenesis during embryogenesis and postnatal muscle regeneration. It has been reported that Wnt1/3a-double-knockout
mouse embryos showed impaired myogenesis (Ikeya and Takada, 1998). Interestingly, Polesskaya et al have demonstrated that Wnts can induce myogenic differentiation in muscle resident CD45+ stem cells during muscle regeneration (Polesskaya et al. 2003). In addition, β-catenin has been shown to induce the myogenic differentiation of rat MSCs through up-regulation of myogenic regulatory factors (MRFs) (Shang et al. 2007), and has revealed essential roles for canonical Wnt1, 3a, 5b and 7a in the induction of myogenesis via activation of gene expression of MRFs (Ridgeway et al. 2000). Finally, regarding bone formation, it has previously been shown that activation of Wnt/β-catenin signalling in adult mesenchymal stem cells could promote osteoblast differentiation (Cai et al. 2016b; Zhou et al. 2016) and further more β-catenin knockout mouse have shown impaired bone formation (Hill et al. 2005; Day et al. 2005). It has also been demonstrated that overexpression of canonical Wnt3a could promote osteogenesis (Zhou et al. 2016). In addition, Lrp5 has been shown to increase bone mass in mice, while a knockout mutation has reduced bone mineral density (Saarinen et al. 2007; Boyden et al. 2002; Van Wesenbeeck et al. 2003) and conditional deletion of β-catenin in mouse embryos has resulted in a deficiency of terminally differentiated osteoblasts in mice (Glass et al. 2005; Holmen et al. 2005).
1.17. Hypothesis and aim of the study

The generation of lineage specific cells in particularly osteoblasts, endothelial cells and cardiomyocytes from mesenchymal stem cells is a gradual process where terminally differentiated cells are formed by a cascade of events such as inhibition of a stem cell associated transcription network, followed by activation or inhibition of signalling networks and finally activation of a lineage specific transcriptional network. The utilization of a cocktail of lineage specific transcription factors has been used for direct reprogramming of stem cells. In addition, utilization of epigenetic modification has also been shown to promote this process further. This suggested that a cascade of events such as activation or de-activation of signalling pathways and transcription machinery control stem cell differentiation to finally produce a terminally differentiated cell type. Accordingly, the inception of this project originated by the main hypothesis and specific aim as outlined below.

Main Hypothesis

Nitric oxide signalling pathway regulates cellular differentiation

Aim 1

Investigating the role of nitric oxide signalling pathway on osteogenic differentiation of equine adipose derived stem cells

Aim 2

Investigating the role of nitric oxide signalling pathway on endothelial differentiation of rat bone marrow derived stem cells
**Aim 3**

Investigating the role of nitric oxide signalling pathway on cardiac reprogramming of human dermal fibroblasts

**Aim 4**

Development of non-viral gene transfer vector system for efficient delivery of eNOS into stem cells

**Aim 5**

Analysing the role of CAV-A subdomain on the solubility of CAV-1 protein
1.18. References


80. Day TF, Guo X, Garrett-Beal L, Yang Y. Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell. 2005; 8(5):739–50.


152. https://clinicaltrials.gov/ct2/show/NCT02032004


183. Kinnaird T, Stabile E, Burnett MS, Lee CW, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res. 2004; 94(5):678-85.


Chapter 2: General materials and methods
2.0. Materials and methods

2.1. Cell culture: Human embryonic kidney 293T, human foreskin fibroblast (BJ) and equine adipose derived mesenchymal stem cells (eASCs)

Human embryonic kidney 293T (HEK293T) cells and human foreskin fibroblast (BJ) (American Type Culture Collection; ATCC, VA, USA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Life technologies) and 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen, CA, USA) (growth medium) in a humidified incubator supplemented with 5% CO2. Cells were passaged with TrypLE™ select (Gibco, NY, USA) when they reached 80-90% confluency.

2.2. Isolation of rat bone marrow-derived mesenchymal stem cells (rBMSCs)

All procedures involving animals were performed in accordance with the ethical regulations of Charles Sturt University (Wagga Wagga, NSW, Australia) and St. Vincent’s Hospital (Melbourne, Victoria, Australia). rBMSCs were isolated from the bone marrow of 4-8 week old male Sprague Dawley rats (Animal Resources Centre, Perth, Australia). Briefly, after euthanasia, marrow was flushed from femoral and tibial compartments with complete rBMSC isolation medium, which consisted of α-MEM (Thermo Fisher Scientific), supplemented with 20% FBS and 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen, CA, USA). Recovered cell suspensions were pooled and centrifuged at 1,200 rpm for 5
min followed by counting and plated at a density of $1 \times 10^6$ cells per cm$^2$. Non-adherent cells were removed after 3 days and medium was changed every 2-3 days. After approximately 8 days or when cell culture had reached confluence, cells were detached enzymatically with TrypLE$^\text{TM}$ select (Gibco, NY, USA) and replated at $6 \times 10^3$ cells per cm$^2$.

2.3. Plasmid preparation, confirmation and cloning

Plasmids used for the generation of lentiviral vectors and minicircle DNA vectors are described in table 2.1. All plasmids were confirmed by DNA sequencing or restriction enzyme digestion, followed by gel electrophoresis analysis.

2.3.1. Restriction enzyme digestion

Restriction enzyme digestion reactions were performed with appropriate restriction enzymes which are described in relevant chapters of this thesis. Briefly, a total reaction mixture volume of 30 µl consisted of, 3 µl of 10X reaction buffer, 1U of restriction enzyme (New England Biolabs, USA) for every 1 µg of plasmid DNA followed by total volume adjustment to 30 µl using deionized H$_2$O. The reaction mixture was then incubated at the recommended temperature by the manufacturer for 1h and the digested DNA was then heat inactivated at 65°C for 20 min and following this, loaded into a 1 % agarose gel for electrophoresis (section 2.3.2).
Table 2.1. The list of plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant properties (transcriptional control/promoters)</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>psPAX2</td>
<td>Lentiviral packaging elements</td>
<td>Addgene (12260)</td>
</tr>
<tr>
<td>pMD2.G</td>
<td>Lentiviral VSV-G envelop</td>
<td>Addgene (12259)</td>
</tr>
<tr>
<td>pRSV-Rev</td>
<td>Lentiviral packaging elements</td>
<td>Addgene (12253)</td>
</tr>
<tr>
<td>pWPT-eNOS</td>
<td>CMV-eNOS</td>
<td>This study</td>
</tr>
<tr>
<td>pLVX-eNOS-GFP</td>
<td>CMV-eNOS-GFP</td>
<td>This study</td>
</tr>
<tr>
<td>FUW-eNOS</td>
<td>TetO-eNOS</td>
<td>This study</td>
</tr>
<tr>
<td>pWPT-CAV-1&lt;sup&gt;T&lt;/sup&gt;SA</td>
<td>CMV-CAV-1&lt;sup&gt;T&lt;/sup&gt;SA</td>
<td>This study</td>
</tr>
<tr>
<td>pLVX-CAV-1&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>CMV-CAV-1&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pTRIP-Runx2-Hsp68-GFP</td>
<td>Runx2.Hsp68-GFP</td>
<td>This study</td>
</tr>
<tr>
<td>pLX304-Wnt3a</td>
<td>CMV-Wnt3a</td>
<td>DNAsu (HsCD00436739)</td>
</tr>
<tr>
<td>pLX304-Wnt5a</td>
<td>CMV-Wnt5a</td>
<td>DNAsu (HsCD00442542)</td>
</tr>
<tr>
<td>TOP-dGFP</td>
<td>TCF/LEF-dGFP</td>
<td>Addgene (14715)</td>
</tr>
<tr>
<td>P-GFP</td>
<td>CMV–MCS–EF1α–GFP</td>
<td>System bioscience (MN511-A1)</td>
</tr>
<tr>
<td>P-eNOS</td>
<td>CMV-eNOS</td>
<td>This study</td>
</tr>
<tr>
<td>FUW-TetO-Gata4</td>
<td>TetO-Gata4</td>
<td>Addgene (41084)</td>
</tr>
<tr>
<td>pLenti6.2/V5-Mef2C</td>
<td>CMV-Mef2C</td>
<td>DNAsu (HsCD00329780)</td>
</tr>
<tr>
<td>pLenti6.2/V5-Tbx5</td>
<td>CMV-Tbx5</td>
<td>DNAsu (HsCD00330096)</td>
</tr>
<tr>
<td>FUW-M2rtTA</td>
<td>Reverse transactivator</td>
<td>Addgene (20342)</td>
</tr>
<tr>
<td>aMHC-GFP</td>
<td>α-MHC-GFP</td>
<td>Addgene (21229)</td>
</tr>
</tbody>
</table>
2.3.2. Agarose gel electrophoresis and gel purification

Agarose gels (1 %) were prepared by melting 1 g agarose (Invitrogen, CA, USA) in 100 ml 1X Tris-base Acetic acid EDTA (TAE) buffer diluted from a 50X TAE (BioRad) in a conical flask by heating in microwave for 2 min. Once melted, 3 μl of ethidium bromide (EtBr) from a 10 mg/ml EtBr stock solution (Sigma-Aldrich) was added to the agarose solution after cooling down to approximately 50°C. The mixture was then poured into a horizontal gel cast electrophoresis apparatus (BioRad) and was allowed to solidify at room temperature for 20 minutes. The agarose gel was then immersed in 1X TAE buffer and loaded with digested DNA or PCR products mixed with of 10X loading buffer (New England Biolabs, USA). Electrophoresis was performed at 60 V for ~80 minutes and DNA bands were visualised under ultraviolet light and images were captured with the BioRad Imaging System. The target fragments were identified and then excised with a clean scalpel blade followed by purification using a QIAgen Gel Extraction kit as instructed by the manufacturer (Qiagen).

2.3.3. General cloning methods

100 ng of the gel purified plasmid DNA and 100 ng of the insert DNA (Gene of interest) were mixed in a 50 µl PCR tube and volume was adjusted to 10 µl using de-ionized water. 10 µl of takara DNA ligation kit (Takara, Japan) was added to the vector-insert mix and incubated at 16°C for 30 min in a thermo cycler (BioRad). The resulted ligation reaction was then used to transform E. coli Top10 super competent cells (Thermo Fisher Scientific).
2.3.4. Bacterial transformation

Transformation of bacteria with plasmids was performed in *E. coli* Top10 super competent cells (Thermo Fisher Scientific). Competent cells were thawed for 2 min on ice and 50 µl of competent cells were mixed with 5 µl of plasmid DNA and incubated on ice for 30 minutes. Heat shock was then performed at 42°C for 40 seconds after which the mixture tube was returned to ice for 5 min to recover from the heat shock. 1 ml of pre-warmed (37°C) sterile LB broth (Difco) was then added to transformed bacterial culture and incubated for 1 h at 37°C with constant shaking at 200 rpm. Following this, 150 µl of the transformed bacterial culture was then spread on sterile LB agar (Difco) containing 100 µg/ml of ampicillin (Sigma-Aldrich) and incubated overnight at 37°C. Single colonies were isolated and plasmid-containing bacteria were grown in LB broth containing 100 µg/ml of ampicillin overnight. Plasmids were then isolated and purified using an Isolate II Plasmid Mini Kit (Bioline) as instructed by the manufacturer. Verification of the plasmids was performed by restriction enzyme digestion or by Sanger sequencing (Australian Genome Sequencing Facility, AGRF).
2.4. Lentiviral vector production

Lentiviral vectors used in this study were generated using the four plasmid transfection system of packaging in HEK293T cells. Briefly, each well of a 6-well tissue culture plate were coated with 50 µg/ml of DL-lysine (Sigma-Aldrich) in PBS for 2 h at 37ºC. HEK293T cells were then seeded at a density of 1 x 10^6 cells per well for 24 h prior to transfection with 6.3 µg of packaging plasmid psPAX2 (Addgene; table 2.1), 3.1 µg of Rev expression plasmid pRSV Rev (Addgene; table 2.1), 3.5 µg of VSV-G envelope expression plasmid pMD2.G (Addgene; table 2.1), and 10 µg of the gene of interest expression transfer vector using a standard calcium phosphate transfection method (Tiscornia et al. 2006). Seventeen hours post-transfection, the growth medium was changed and supernatant containing lentiviral particles was collected at 48 h and 72 h post-transfection, combined, and filtered through a 0.45-µM PVDF filter, and concentrated using PEG-it Virus Precipitation Solution (System Biosciences) and stored at -80 until used.

2.5. Gene expression (mRNA transcript) analysis

2.5.1. RNA extraction

RNA extraction was performed using TriReagent (Thermo Fisher Scientific) from cultured mammalian cells, following the manufacturer’s protocol. Briefly, 0.5 ml of TriReagent was added to each well of 12-well tissue culture plate to disrupt the cell membrane and organelles and release the total RNA contained in cells. The lysate was then transferred into a 1.5 ml eppendorf tube and was vigorously mixed with 100 µl of chloroform (Sigma-Aldrich) followed by incubation at room temperature
for 15 min. The mixture was then centrifuged for 15 minutes at 12,000 x g at 4°C and the upper aqueous layer was transferred without disturbing the bottom organic layer. To precipitate RNA, 0.25 ml of isopropanol was added, vortexed, and incubated for 10 min at room temperature. Following this, the RNA was precipitated by centrifuging at 12,000 x g for 15 min and the recovered RNA pellet was washed with 75% ethanol and air dried for 5 min and re-suspended in 30 µl of RNase free water (Qiagen). RNA concentration was measured using a Nanodrop Spectrophotometer (Thermo Fisher Scientific). RNA was then treated with RQ1 RNase free DNase (1 U/1 µg RNA; Promega, WI, USA) according to the manufacturer’s protocol followed by incubation at 65°C for 10 min to inactivate DNase and was then stored at -80°C.

2.5.2. Reverse transcription (RT) of RNA to obtain cDNA

cDNA was synthesized from 1 µg of total RNA for each reverse transcription (RT) reaction with a high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). The RT reaction was performed in a thermal cycler with 10 µl total reaction mixture, in which, 1 µL 10X RT buffer, 0.4 µL 25X dNTP mix (100 mM), 1 µL 10X RT random primers, 2.1 µL nuclease free water, 0.5 µL multiscrize reverse transcriptase (added last to the mixture). The mixture was then incubated at 25°C for 5 min, 37°C for 2 h and 85°C for 5 min and finally cool down to 4°C. The resulting cDNA was diluted with nuclease free water to obtain a final concentration of 50 ng/ µl and stored at -20°C.
2.5.3. Conventional polymerase chain reaction (PCR)

PCR was carried out in a thermal cycler (BioRad) with 50 µl total reaction volume, in which 25 µl 2X GoTaq PCR master mix (Promega) was combined with 50 ng of cDNA, 1 µl of forward and reverse primer (each from a 100 µM stock) followed by total volume adjustment to 50 µl using de-ionized water. The mixture was then loaded into PCR tubes and amplified with the reaction conditions as shown below in table 2.2. Primers specific to the target products were designed using the Primer-BLAST tool at NCBI. All primers were synthesized (Sigma-Aldrich), re-suspended in deionized water, aliquoted and stored at -20°C.

Table 2.2. PCR conditions

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Incubation time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 S</td>
<td>30</td>
</tr>
<tr>
<td>Primer annealing</td>
<td>60°C</td>
<td>30 S</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Holding</td>
<td>4°C</td>
<td>α</td>
<td>1</td>
</tr>
</tbody>
</table>

2.5.4. Quantitative real time polymerase chain reaction (RT-qPCR)

Relative gene expression was detected by RT-qPCR using the BioRad master mix. 20 ng of cDNA were combined with 5 µl of 2X SsoFast EvaGreen Supermix (BioRad) and 0.1 µl of forward and reverse primers (each from 100 µM stock) for
the target gene and the final volume was adjusted to 10 µl using de-ionized water. The samples were then loaded onto 96 well plates (BioRad, real-time PCR plates) and the reaction conditions were 5 min at 95°C and this was followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds followed by 10 minutes of 95°C to establish the melting curve of the primers. The threshold cycle (Ct) values were automatically obtained in excel format and target mRNA expression was normalized to β-actin or GAPDH Ct values which served as the internal endogenous controls. For every sample, RT-qPCR was performed in triplicate and the primers were designed using the Primer-BLAST tool software provided by NCBI. The primer sequences are listed in each chapter and used for the relevant study.

2.6. *In vivo* experimentation

2.6.1. Scaffold preparation

The genetically modified rBMSCs (see the sections of 4.3.5 and 4.3.6 in chapter 4) were resuspended in 25 µl of fibrinogen (30 mg/ml, Sigma Aldrich) and this was followed by addition of 5 µl of thrombin (1U/µl Sigma Aldrich). The cell suspensions were then seeded on 6 mm polyurethane scaffolds (PolyNovo, Australia, kindly provided by Dr Geraldine Mitchell at St. Vincent’s Institute of Medical Research) and incubated for 5 min at room temperature followed by incubation in growth medium for 1 h before transplantation.
2.6.2. *In vivo* subcutaneous transplantation

Male nude rats were purchased from Animal Resources Centre (Perth, Australia) and maintained with a 12 hour light/dark cycle and given water and food. Experimental procedures were approved by St. Vincent’s Hospital, Melbourne Animal Ethics Committee.

2.6.3. Aseptic technique

To maintain sterile conditions, all surgical equipment was autoclaved at 125°C for 15 min. Animals were given Baytril as antibiotic in drinking water (0.175 mg/ml) for three days before and three days after surgery.

2.6.4. Surgery and scaffold transplantation

The detailed procedure for the surgery is described in section 4.3.14 in chapter 4 and the histological analysis (tissue fixation, processing, and paraffin embedding) was also described in section 4.3.15.1 in chapter 4.

2.6.5. Heamatoxylin and Eosin (H&E) staining

For general tissue morphology, heamatoxylin and eosin (H&E) staining was performed as shown below in table 2.3.
<table>
<thead>
<tr>
<th>Reagents</th>
<th>Duration</th>
<th>Indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hostolene</td>
<td>5 minutes</td>
<td>Dewax</td>
</tr>
<tr>
<td>Hostolene</td>
<td>5 minutes</td>
<td>Dewax</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>1 minute</td>
<td>Hydrate</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>70% ethanol</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>Wash slides three times in tap water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mayer’s hematoxylin</td>
<td>5 minutes</td>
<td>Nuclear stain (purple/blue)</td>
</tr>
<tr>
<td>Wash slides three times in tap water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonia water</td>
<td>1 minute</td>
<td>Bluing agent</td>
</tr>
<tr>
<td>Wash slides one in tap water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosin</td>
<td>2 minutes</td>
<td>Cytoplasm and other tissue elements stain (Pink)</td>
</tr>
<tr>
<td>Wash slides three times in tap water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70% ethanol</td>
<td>1 minute</td>
<td>Dehydrate</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>100% ethanol</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>Histolene</td>
<td>2 minutes</td>
<td>Clear</td>
</tr>
<tr>
<td>Histolene</td>
<td>2 minutes</td>
<td></td>
</tr>
<tr>
<td>Add DPX mounting media, coverslip, and dry overnight.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.7. Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using the Graphpad Prism software. See individual results chapter for detail statistical analysis. A value of $p<0.05$ was considered statistically significant.

2.8. References

Chapter 3: Molecular control of nitric oxide synthesis through eNOS and caveolin-1 interaction regulates osteogenic differentiation of adipose derived stem cells by modulation of Wnt/β-catenin signalling

Article published in *Stem Cell Research and Therapy* and attached in appendix 3 (pp 385-399). This article has been highlighted in “Mesenchymal Cell News” (https://www.mesenchymalcellnews.com/issue/volume-8-49-dec-13/)
3.1. Abstract

Nitric oxide (NO) plays a role in a number of physiological processes including stem cell differentiation and osteogenesis. Endothelial nitric oxide synthase (eNOS), one of three NO-producing enzymes, is located in a close conformation with the caveolin-1 (CAV-1WT) membrane protein which is inhibitory to NO production. Modification of this interaction through mutation of the caveolin scaffold domain can increase NO release. In this study, equine adipose-derived stem cells (eASCs) were genetically modified with eNOS, CAV-1WT, and a CAV-1F92A (CAV-1WT mutant) and assessed NO-mediated osteogenic differentiation and the relationship with the Wnt signaling pathway. In results, NO production was enhanced by lentiviral vector co-delivery of eNOS and CAV-1F92A to eASCs, and osteogenesis and Wnt signaling was assessed by gene expression analysis and activity of a novel Runx2-GFP reporter. Cells were also exposed to a NO donor (NONOate) and the eNOS inhibitor, L-NAME. NO production as measured by nitrite was significantly increased in eNOS and CAV-1F92A transduced eASCs (5.59 ± 0.22 μM) compared to eNOS alone (4.81 ± 0.59 μM) and un-transduced control cells (0.91 ± 0.23 μM) (p < 0.05). During osteogenic differentiation, higher NO correlated with increased calcium deposition, Runx2, and alkaline phosphatase (Alp) gene expression and the activity of a Runx2-eGFP reporter. Co-expression of eNOS and CAV-1WT transgenes resulted in lower NO production. Canonical Wnt signaling pathway-associated Wnt3a and Wnt8a gene expressions were increased in eNOS-CAV-1F92A cells undergoing osteogenesis whilst non-canonical Wnt5a was decreased and similar results were seen with NONOate treatment. Treatment
of osteogenic cultures with 2 mM L-NAME resulted in reduced Runx2, Alp, and Wnt3a expressions, whilst Wnt5a expression was increased in eNOS-delivered cells. Co-transduction of eASCs with a Wnt pathway responsive lenti-TCF/LEF-dGFP reporter only showed activity in osteogenic cultures co-transduced with a doxycycline inducible eNOS. Lentiviral vector expression of canonical Wnt3a and non-canonical Wnt5a in eASCs was associated with induced and suppressed osteogenic differentiation, respectively, whilst treatment of eNOS-osteogenic cells with the Wnt inhibitor Dkk-1 significantly reduced expressions of Runx2 and Alp. In summary, this study identifies NO as a regulator of canonical Wnt/β-catenin signaling to promote osteogenesis in eASCs which may contribute to novel bone regeneration strategies.
3.2. Introduction

Mesenchymal stem cells (MSCs) have been isolated from various tissues such as adipose (Zannettino et al. 2008) heart (Zhang et al. 2015), bone marrow (Friedenstein et al. 1968; Bandara et al. 2016), and blood (He et al. 2007; Oh et al. 2008; Meng et al. 2007; Hida et al. 2008; Patel et al. 2008), and have the potential to differentiate into different lineages, including osteoblasts, chondrocytes, and adipocytes (Prockop et al. 1997; Phinney et al. 2007). The osteoblast differentiation program of MSCs is switched on by cell recruitment, and timely expression of genes including Runx2, alkaline phosphatase (Alp), type I collagen (ColA1), and osteocalcin (OC) followed by extracellular matrix mineralization (Lian et al. 2004). This process can be induced by soluble molecules such as bone morphogenetic proteins (BMPs) (Zhang et al. 2014) or Wnts (Gaur et al. 2005; Choi et al. 2015; Goodnough et al. 2014) that activate several pathways and other various downstream signals such as protein kinase (Ge et al. 2007) and growth factors (Kratchmarova et al. 2005) to trigger osteoblast differentiation of mesenchymal stem cells.

Nitric oxide (NO) is a signaling molecule with a short half-life (Ignarro et al. 1987; Vaughn et al. 1998). It can react within the cell where it is produced or penetrate cell membranes to affect adjacent cells (Lancaster Jr, 1997). NO exerts a variety of physiological effects such as regulating blood pressure via smooth muscle relaxation (Tare et al. 1990), mediating immune responses (Bogdan, 2001), controlling cell proliferation (Davis et al. 2001), modulating apoptosis (Vaughn et al., 1998), promoting growth factor-induced angiogenesis (Bandara et al. 2016;
Lancaster Jr, 1997), accelerating wound healing (Bandara et al. 2016; Clark et al. 2000), and functioning as a neurotransmitter (Miller et al. 2009). These responses can be mediated through activating the primary NO effector soluble guanylyl cyclase to produce cGMP (Arnold et al. 1977) by NO-based chemical modifications of proteins through S-nitrosylation (Hess et al. 2005) or through epigenetic modification (Hickok et al. 2013). NO is known to play an important role in bone homeostasis. It is generated by many cell types present in the bone environment, most notably the osteoblast (Evans and Ralston, 1996).

NO is synthesized from L-arginine by three isozymes of nitric oxide synthase (NOS), including neuronal NOS (nNOS), endothelial NOS (eNOS), and cytokine-inducible NOS (iNOS) (Thomas, 2015). Both iNOS (Lin et al. 2007; Hou et al. 2009) and eNOS (Ma et al. 2014) have been shown to play a role in osteoblast differentiation. Mice lacking eNOS have shown marked bone abnormalities due to impaired osteoblast differentiation resulting in poor maintenance of bone mass (Aguirre et al. 2001; Armour et al. 2001). Gene expression data from neonatal calvarial osteoblasts from eNOS−/− mice have shown downregulation of Runx2, Cbfa-1, and osteocalcin (Afzal et al. 2004). On the other hand, high concentrations of NO released due to the pathological iNOS expression promote bone resorption through induced osteoclastogenesis (Zhao et al. 2015). Therefore, an optimum level of NO is important to drive osteogenic differentiation of the MSCs.

In contrast with other NOS family members, eNOS is localized mainly in specific intracellular membrane domains, including the Golgi apparatus (Iwakiri et al. 2006) and plasma membrane caveolae (Garcia-Cardena et al. 1996; Bernatchez et al.
A previously demonstrated direct interaction of eNOS with wild-type caveolin-1 (CAV-1\(^{WT}\)) (Feron et al. 1996) has proposed that CAV-1\(^{WT}\) functions as an endogenous negative regulator of eNOS (Maniatis et al. 2008). In this context, eNOS binds to the caveolin-1 scaffolding domain (CSD; amino acids 82–101) (Garcia-Cardena et al. 1997) and, furthermore, Thr-90 and Thr-91 (T90 and T91), and Phe-92 (F92) were identified as critical residues for eNOS binding and inhibition (Bernatchez et al. 2005). Genetic modification of endothelial cells through overexpression of a mutated version of CAV-1 with a phenylalanine to alanine substitution at the amino acid position 92 (CAV-1\(^{F92A}\)) resulted in increased NO production, overcoming the inhibitory effect of CAV-1\(^{WT}\) (Bernatchez et al. 2005).

This study tested the hypothesis that molecular control of NO synthesis in equine adipose-derived stem cells (eASCs), can promote osteogenic differentiation where endogenous eNOS is not available, by recreating the interaction between eNOS and CAV-1 (CAV-1\(^{WT}\) and CAV-1\(^{F92A}\)) regulates the osteogenic differentiation of eASCs. Results from this study indicate that the optimum level of NO induces osteogenic differentiation through activation of the downstream canonical Wnt/β-catenin signaling pathway.
3.3. Materials and methods

3.3.1. Cell culture
eASCs were isolated from subcutaneous adipose tissue as previously described (Petersen et al. 2014). All sampling was carried out using protocols approved by the Charles Sturt University Animal Care and Ethics Committee. Human embryonic kidney 293 T cells (HEK293T) (ATCC, VA, USA) and eASCs were cultured and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Bovogen, VIC, Australia), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen) (growth medium) at 37 °C and 5% CO₂.

3.3.2. Plasmid constructs
The list of cDNA for the genes of interest used in this study is listed in Table 3.1, and was used for construction of lentiviral vectors. HF Phusion (New England Biolabs; NEB) DNA polymerase was used for all the polymerase chain reactions (PCRs) and all the restriction endonucleases were purchased from NEB unless indicated otherwise.

To construct the CMV promoter-driven eNOS expressing lentiviral vector, a codon optimized eNOS gene was synthesized (Chapter 6 [Bandara et al. 2016]) and subcloned into the pWPT-GFP lentiviral plasmid (Addgene, MA, USA) using BamH1 and SalI restriction endonucleases. Doxycycline (DOX) inducible eNOS construct was prepared by amplifying the eNOS gene using the forward
(ATCAGAATTCTGACAGCTGGAAGCTGGA) and reverse primer (ATCAGAATTCTCAGGCTGGA) by introducing EcoR1 restriction sites (underlined sequences in both forward and reverse primers) at both the 5' and 3' ends of the final PCR product, followed by subcloning the PCR product into FUW-TetO vector (Addgene). Human wild-type caveolin-1 (CAV-1 WT) expressing lentiviral vector was constructed by inserting the full length CAV-1 WT (Addgene) into pLVX-AcGF1-C1 and pLVX-DsRed-C1 (Clontech, CA, USA) using EcoR1 and BamH1 restriction endonucleases. A mutated caveolin-1 (CAV-1 F92A) in which phenylalanine (F) at the amino acid position 92 was replaced with alanine (A) (Bernatchez et al., 2005) was synthesized (Geneart), amplified by PCR by introducing BamH1 and SalI sites at the 5' and 3' ends of the PCR product, respectively, via forward primer (ATCAGGATCCATGTCTGGGGGCAT) and reverse primer (ATCAGTCGACTTTATATTTCTTTCTG) (restriction sites are underlined). The PCR product was then ligated into the pWPT-GFP lentiviral vector (Addgene) at the BamH1 and SalI restriction sites replacing GFP. Wnt3a and Wnt5a expressing lentiviral plasmids were purchased from DNAsu plasmid repository.

3.3.3. GFP reporter constructs

A 343-bp fragment of the Runx2 enhancer region (sequence information was kindly provided by Toshihisa Komori at the Department of Cell Biology, Nagasaki University) (Kawane et al. 2014) was synthesized together with the sequence of the Hsp68 minimal promoter (GenScript, NJ, USA). The entire fragment was then subcloned into pTRIP-eGFP lentiviral vector at the Mlu1 and BamH1 restriction sites.
sites replacing a rat insulin-specific promoter (RIP), upstream of the enhanced GFP (eGFP) coding sequence. The Wnt responsive lentiviral TCF/LEF-dGFP reporter system was purchased from Addgene.

3.3.4. Lentiviral vector production and transduction of equine adipose stem cells

Lentiviral vectors used in this study (Table 3.1) were generated by four plasmid transfection of HEK293T cells. Briefly, each well of a six-well tissue culture plate was coated with 50 μg/ml of DL-lysine (Sigma-Aldrich) in phosphate-buffered saline (PBS) and incubated for 2 h at 37 °C. HEK293T cells were then seeded at a density of $1 \times 10^6$ cells per well, 24 h prior to transfection of 6.3 μg of packaging plasmid psPAX2 (Addgene), 3.1 μg of Rev expression plasmid pRSV Rev (Addgene), 3.5 μg of VSV-G envelop pMD2.G (Addgene), and 10 μg of the gene of interest expression transfer vector using a standard calcium phosphate transfection method (Kingston et al. 2003). Seventeen hours post-transfection, the media was changed and supernatant containing lentiviral vectors were collected at 48 h and 72 h post-transfection, combined, and filtered through a 0.45-μM PVDF filter, and used for eASC transduction in the presence of 4 μg/ml Polybrene (Sigma-Aldrich).
3.3.5. Osteogenic differentiation

eASCs were seeded in a 12-well plate (11,000 cells/cm²) in triplicate in growth medium overnight followed by transduction with eNOS, CAV-1WT, and CAV-1F92A lentiviruses. After 3 days, growth medium was replaced with osteogenic induction medium (OM; growth medium+0.2 mM 2-phospho-l-ascorbic acid trisodium salt+10 nM dexamethasone+10 mM β-glycerol phosphate with or without 2 µg/ml Doxycycline). Non-induced control cells were cultured in growth medium. Medium was changed every 3 days (see Fig. 3.2a below).

After 11 days incubation in OM or control growth medium, cells were washed with PBS and fixed with 4% (w/v) paraformaldehyde (Sigma-Aldrich) for 20 min, washed with distilled water, and then stained with 2% (w/v) Alizarin Red S (pH 4.2) for 20 min. Stained cells were washed with distilled water prior to assessment by light microscopy using a Nikon Eclipse Ti-S inverted microscope (Nikon, Japan).
Table 3.1 Lentiviral vectors and reporter constructs used in this study

<table>
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<tr>
<th>Lentiviral vector</th>
<th>Relevant properties</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWPT-eNOS</td>
<td>CMV-eNOS</td>
<td>This study</td>
</tr>
<tr>
<td>FUW-eNOS</td>
<td>TetO-eNOS</td>
<td>This study</td>
</tr>
<tr>
<td>pWPT-Cav-1&lt;sup&gt;Tmca&lt;/sup&gt;</td>
<td>CMV-Cav-1&lt;sup&gt;Tmca&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Runx2.Hsp68-eGFP</td>
<td>This study</td>
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<tr>
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<td>CMV-Wnt3a</td>
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<td>DNASU (HsCD00442542)</td>
</tr>
<tr>
<td>pRRL-TCF/LEF-GFP</td>
<td>TCF/LEF-dGFP</td>
<td>Addgene (#14715)</td>
</tr>
<tr>
<td>Reverse tetracycline transactivator</td>
<td>FUW-M2rtTA</td>
<td>Gift from Rudolf Jaenisch (Addgene plasmid # 20342)</td>
</tr>
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</table>

3.3.6. Alizarin Red S quantification

Quantification of Alizarin Red S staining was performed as previously described (Gregory et al. 2004). Briefly, after staining the cells with Alizarin Red S for 20 min, 10% acetic acid was added to the 12-well cell culture plate and incubated for 30 min with shaking. The Alizarin Red S stain was extracted and the absorbance was measured at 405 nm in parallel with Alizarin Red S standards comprising of serial 1:2 dilutions of 50 mM Alizarin Red S (pH 4.2). Standard curve (from 0.1 µM to 10 µM) was prepared. .
3.3.7. Quantitative real time PCR

Total RNA from transduced and control cells after 11 days of incubation in OM or growth medium was isolated using the PureZol reagent (Bio-Rad, CA, USA) according to the manufacturer’s instructions, and the concentration of isolated RNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific), treated with RQ1 RNase free DNase (1 U/1 μg RNA; Promega, WI, USA). cDNA was synthesized with 1 μg RNA from all samples using a High Capacity Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative real-time PCR assays were performed on a BioRad CFX96 Real-Time system (Bio-Rad) using the SsoFast EvaGreen Supermix (Bio-Rad). Primer sequences used for target gene amplification are described in Table 3.2. Assays were performed in triplicate and target gene expression was normalized to equine β-actin mRNA levels using the ΔΔC_t method.
<table>
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<th>Reverse (5’ &gt; 3’)</th>
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</tr>
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</table>

3.3.8. Immunocytochemistry and confocal microscopy

Immunocytochemical detection of eNOS and caveolin-1 (CAV-1<sup>WT</sup> and CAV-1<sup>F92A</sup>) expression in eASCs was performed as follows. Briefly, cells were fixed in 4% paraformaldehyde for 20 min at 37°C, treated with 0.1% Triton-X100 in PBS for 10 min, and blocked in a 10% FBS in PBS solution for 30 min at room temperature. This was followed by a 2 h incubation with a primary mouse monoclonal anti-eNOS antibody (1:100; BD Biosciences, CA, USA) or rabbit polyclonal anti-CAV-1 antibody (1:100; Cell Signaling Technology, MA, USA), and subsequently with an anti-mouse IgG secondary antibody conjugated with Alexa 488 (1:200; Cell Signaling Technology) or anti-rabbit IgG secondary antibody conjugated with Alexa...
555 (1:200; Cell Signaling Technology) for 1 h and counterstained with DAPI for nuclear staining (Sigma-Aldrich). eNOS and CAV-1 co-localization was observed by confocal microscopy (Nikon).

To detect β-catenin expression, eNOS transduced cells (with or without DOX treatment) and un-transduced cells were fixed in 4% paraformaldehyde for 20 min at 37 °C, treated with 0.1% Triton-X100 in PBS for 10 min, and blocked in a 10% FBS in PBS solution for 30 min at room temperature. This was followed by an overnight incubation with a primary rabbit monoclonal anti-β-catenin antibody (1:100; Cell Signaling Technology) and subsequently with an anti-rabbit IgG secondary antibody conjugated with Alexa 488 (1:200; Cell Signaling Technology) for 1 h and counterstained with DAPI.

3.3.9. GFP Reporter assays

For GFP-based reporter assays for both TCF/LEF-dGFP and Runx2.Hsp68-eGFP, cells transduced with the TCF/LEF-dGFP and Runx2.Hsp68-eGFP were subjected for reverse transcription quantitative PCR (RT-qPCR) for GFP expression and fluorescence microscopic analysis, respectively.

3.3.10. Nitrite level detection

Extracellular nitrite level was measured using the Griess reagent (Promega) according to the manufacturer’s instructions and measurement of absorbance at 540 nm. Triplicates of each sample were measured at each time-point during
osteogenic differentiation from day 0 to day 11. Standard curve (from 0.1 µM to 100 µM) was prepared with standard provided by the manufacture in the cell culture medium (DMEM).

3.3.11. Statistical analysis

All experiments were performed in triplicate and at least three times. Data are presented as mean ± SEM. The statistical significances were determined by one-way analysis of variance (ANOVA) followed by post hoc. All tests were performed using the statistical software GraphPad Prism 6 (GraphPad, CA, USA) and $p < 0.05$ was considered statistically significant.
3.4. Results

3.4.1. eASC characterization

eASCs were spindle-shaped and adherent to plastic tissue culture dishes (data not shown) and it has previously been reported their tri-lineage differentiation potentials (Petersen et al. 2015).

3.4.2. eNOS and caveolin-1 expression in eASCs

eNOS activation is controlled at the cell plasma membrane significantly by CAV-1, a major structural protein in caveolae (Bucci et al. 2000; Michel et al. 1997). Expression of eNOS and CAV-1 was investigated in un-transduced and transduced eASCs by immunofluorescence microscopy. Wild-type CAV-1 (CAV-1\textsuperscript{WT}) transduced eASCs (eASC\textsuperscript{CAV-1WT}) and un-transduced eASCs (eASC\textsuperscript{WT}) expressed CAV-1 protein; notably, the CAV-1 expression was increased in eASC\textsuperscript{CAV-1WT} (Fig. 3.1d) compared to eASC\textsuperscript{WT} (Fig. 3.1c). Interestingly, eNOS expression was absent in eASC\textsuperscript{WT} (Fig. 3.1a), whereas strong eNOS expression was observed in eNOS-transduced cells (eASC\textsuperscript{eNOS}) (Fig. 3.1b). Next, the localization of eNOS, CAV-1\textsuperscript{WT}, and mutated CAV-1 (CAV-1\textsuperscript{F92A}) in genetically modified eASC was investigated by confocal microscopy. As expected, eNOS expression was detected at the cytoplasm (Fig. 3.1e), whereas both the CAV-1\textsuperscript{F92A} (Fig. 3.1f) and CAV-1\textsuperscript{WT} (Fig. 3.1g) expressions were observed at the plasma membrane, confirming that F92A mutation of CAV-1 does not affect its cellular localization. Co-localization of eNOS and CAV-1\textsuperscript{F92A} in co-transduced eASCs with
eNOS and CAV-1$^{F92A}$ (eASC$^{eNOS+CAV-1F92A}$) was examined by confocal microscopy with expression of eNOS in the cytoplasm and CAV-1$^{F92A}$ at the plasma membrane (Fig. 3.1h). As controls for primary antibodies, immunostaining was carried out in the absence of primary antibodies specific to eNOS and CAV-1 (Figure 3.S1).
Figure 3.1. Immunofluorescence localization of eNOS, CAV-1\textsuperscript{F92A}, and CAV-1\textsuperscript{WT} in lentiviral transduced eASCs and un-transduced cells. 

a Un-transduced eASCs show no endogenous endothelial nitric oxide synthase (eNOS) expression. 
b eNOS-transduced eASCs (green) show strong cytoplasmic expression. 
c Un-transduced eASCs show endogenous caveolin expression (red). 
d Wild-type caveolin-1 (CAV-1\textsuperscript{WT}) transduced eASCs show significantly stronger expression. 

When e eNOS, f mutated caveolin-1 (CAV-1\textsuperscript{F92A}), and g CAV-1\textsuperscript{WT} were transduced to eASCs using lentiviral vectors, eNOS showed cytoplasm localization (red) whereas both the CAV-1\textsuperscript{F92A} and CAV-1\textsuperscript{WT} showed plasma membrane localization (green). 

h Confocal microscopy analysis of co-transduction of eASCs with eNOS and CAV-1\textsuperscript{F92A} resulted in cytoplasmic eNOS expression (red) and membrane localisation of CAV-1\textsuperscript{F92A} (green).
Figure 3.S1. As a control for specific primary antibody binding, immunostaining was performed in the absence of primary antibodies, with only fluorophore tagged secondary antibody present. Absence of fluorescence signals was detected these controls for eNOS and CAV-1. (a) Detection of nuclear staining by Dapi and (b) immunostaining performed without mouse monoclonal anti-eNOS antibody but with an anti-mouse IgG secondary antibody conjugated with Alexa 488. (c) Detection of nuclear staining by Dapi and (d) immunostaining performed without rabbit polyclonal anti-CAV-1 antibody but with an anti-rabbit IgG secondary antibody conjugated with Alexa 555. (e) Detection of nuclear staining by Dapi and (d) fluorescence detection with no mouse monoclonal anti-eNOS antibody with an anti-mouse IgG secondary antibody conjugated with Alexa 555. (f) Detection of nuclear staining by Dapi and (g) fluorescence detection with no rabbit polyclonal anti-CAV-1 antibody with an anti-rabbit IgG secondary antibody conjugated with Alexa 488.
3.4.3. NO enhances osteogenic differentiation

To examine the role of the NO signaling in eASCs osteogenesis, first, the osteogenic differentiation between eNOS transduced (eASC\textsuperscript{eNOS}) and untransduced eASCs (eASC\textsuperscript{WT}) was compared. A greater number of Alizarin Red S-positive nodules were induced in the eASC\textsuperscript{eNOS} cultures compared to eASC\textsuperscript{WT} culture after 11 days (Fig. 3.2b). NO synthesis was also significantly increased in eASC\textsuperscript{eNOS} compared to eASC\textsuperscript{WT} (Fig. 3.2c). Quantification of calcium deposition showed increased levels of calcium deposition in eASC\textsuperscript{eNOS} compared to eASC\textsuperscript{WT} (Fig. 3.2d). Quantitative analysis of Runx2 (Fig. 3.2e) and Alp (Fig. 3.2f) gene expression were also significantly upregulated in the eASC\textsuperscript{eNOS} cultures compared to the eASC\textsuperscript{WT} cultures.

NO-mediated osteogenic differentiation was further highlighted by inhibition of eNOS activity. eASC\textsuperscript{eNOS} were treated with 2 mM of the nitric oxide synthase inhibitor, \textit{L}-\textit{N}\textsuperscript{G}-nitroarginine methyl ester (\textit{L}-NAME) for 11 days. \textit{L}-NAME treatment resulted in a significant down regulation of osteoblast-specific marker expressions, Alp (Fig. 3.3a) and Runx2 (Fig. 3.3b), compared to untreated eASC\textsuperscript{eNOS}.

3.4.4. Co-expression of eNOS and CAV-1\textsuperscript{F92A} enhances NO production and osteogenic differentiation

Lentiviral vectors expressing eNOS and CAV-1\textsuperscript{F92A} (mutant) or CAV-1\textsuperscript{WT} (wild-type) were co-expressed in eASCs, eASC\textsuperscript{eNOS+CAV-1\textsuperscript{F92A}}, and eASC\textsuperscript{eNOS+CAV-1\textsuperscript{WT}}, respectively. Co-expression of eNOS and CAV-1\textsuperscript{F92A} promoted osteogenesis as
evident by Alizarin Red S staining (Fig. 3.2b) and calcium deposition (Fig. 3.2d) compared to eNOS alone (eASC<sup>enOS</sup>), and NO levels were also significantly increased in the eASC<sup>enOS+CAV-1F92A</sup> (Fig. 3.2c). Co-expression of eNOS with CAV-1<sup>WT</sup> in eASCs (eASC<sup>enOS+CAV-1WT</sup>) reduced NO production (Fig. 3.2c) and also osteogenesis as evident by Alizarin Red staining (Fig. 3.2b) and calcium deposition (Fig. 3.2d). Quantitative real-time PCR analysis revealed that Runx2 (Fig. 3.2e) and Alp (Fig. 3.2f) were significantly up regulated in the eASC<sup>enOS+CAVF92A</sup> cultures and downregulated in the eASC<sup>enOS+CAV-1WT</sup> cultures as compared with the eASC<sup>enOS</sup> cultures (Fig. 3.2e and f).
a) Adipose derived stem cells (eASCs) transduction with lent eNOS, CAV-1WT, CAV-1TMD
Switch to osteogenic induction medium
RNA extraction and Alizarin red staining

b) Growth medium Osteogenic induction medium
Un-transduced Un-transduced eNOS eNOS + CAV-1TMD
Osteogenic induction medium eNOS + CAV-1WT CAV-1TMD CAV-1WT

(c) Nitrite Concentration (μM) vs Time (Days)

(d) Calcium Concentration (μM)

(e) Runx2 Relative mRNA Expression

(f) Alp Relative mRNA Expression

Osteogenic induction - + + + + + +
Figure 3.2. Nitric oxide promotes osteogenic differentiation of eASCs. a) Schematic of lentiviral transduction and osteogenic induction strategy. b) Alizarin Red S staining after 11 days in osteogenic induction medium or growth medium. c) Nitric oxide release from cells undergoing osteogenic differentiation through quantification of nitrite by Greiss assay. d) Quantification of calcium deposition. Relative mRNA transcripts analysis by qPCR of e) Runx2 and f) alkaline phosphatase (Alp) osteoblast markers showing increased expressions in endothelial nitric oxide synthase (eNOS) and eNOS+mutated caveolin-1 (eNOS+CAV-1 F92A) transduced cells compared to all other treatments. *p<0.05, versus eNOS+CAV-1 WT, CAV-1 WT, CAV-1 F92A, eASC (osteogenic induction), eASC (growth medium); † p<0.05 versus eNOS, eNOS+CAV-1 WT, CAV-1 WT, CAV-1 F92A, eASC (osteogenic induction), eASC (growth medium). One way ANOVA followed by post hoc. N=3
Figure 3.3. Inhibition of endothelial nitric oxide synthase (eNOS) through \( \text{L-NAME} \) treatment down regulates eASC osteogenic differentiation. Relative mRNA transcripts analysis by qPCR showing that 2 mM \( \text{L-NAME} \) treatment decreased the expression of osteoblast markers a alkaline phosphatase (Alp) and b Runx2 in eNOS transduced eASCs compared to untreated eNOS transduced cells. *\( p<0.05 \), versus eNOS (\( \text{L-NAME} \)), eASC (osteogenic induction), and eASC (growth medium). One way ANOVA followed by post hoc. N=3
3.4.5. Exogenous NO donor enhances osteogenesis of eASC in a dose-dependent manner

To confirm the direct role of NO levels on eASC osteogenesis, eASCs were treated with a concentration range of exogenous NO donor (NONOate; Sigma-Aldrich). Treatment with exogenous NO donor promoted osteogenesis from 5 μM to 15 μM but this was reduced with high concentrations of NO donor (20 μM) as evident by Alizarin Red staining (Fig. 3.4a). NONOate treatment also resulted in a dose-dependent increase in Runx2 (Fig. 3.4b) and Alp (Fig. 3.4c) gene expression, in which maximum levels of both were achieved with 15 μM NONOate; notably, significantly lower levels of Runx2 and Alp expression were observed with 20 μM NO donor treatment.
Figure 3.4. The exogenous nitric oxide donor NONOate promotes osteogenic differentiation in a dose-dependent manner. a) Alizarin Red S staining of eASCs after 11 days in osteogenic induction medium or growth medium. NO donor concentrations are embedded in the each figure. Relative mRNA transcript analysis shows a significant increase of osteoblast markers b) Runx2 and c) alkaline phosphatase (Alp) when the eASCs were treated with 15 μM NONOate. *p<0.05, versus 0 μM, 5 μM, 10 μM, and 20 μM NONOate, and control (growth medium). One way ANOVA followed by post hoc. N=3
3.4.6. NO promotes endogenous Runx2 expression in differentiating eASCs

To monitor the effect of NO on endogenous Runx2 expression in differentiating eASCs, a GFP lentiviral reporter system was generated under the control of a Runx2 enhancer fused to the Hsp68 promoter (Runx2.Hsp68-eGFP; Fig. 3.5a) based on a novel Runx2 enhancer. eASCs which were stably transduced with the Runx2 reporter showed low levels of GFP expression in mostly undifferentiated cells (Fig. 3.5b), whereas GFP expression as a result of Runx2 promoter activity in differentiating eASCs was increased (Fig. 3.5b). When eASCs were transduced with eNOS (eASC<sup>eNOS</sup>), the GFP signals were increased compared to eASCs co-transduced with eNOS and CAV-1<sup>WT</sup> (eASC<sup>eNOS+CAV-1WT</sup>) and un-transduced control (eASC<sup>WT</sup>) (Fig. 3.5b). Interestingly, endogenous Runx2 activity was remarkably increased when the eASCs were co-transduced with eNOS and CAV-1<sup>F92A</sup> (eASC<sup>eNOS+CAV-1F92A</sup>) (Fig. 3.5b) compared to eASC<sup>eNOS</sup>, eASC<sup>eNOS+CAV-1WT</sup>, and eASC<sup>WT</sup>. Osteogenic nodule formation was significantly increased in the eASC<sup>eNOS+CAV-1F92A</sup> (Fig. 3.5c), and these result suggests that endogenous Runx2 expression is less active in undifferentiated eASCs and its expression is significantly increased through NO signaling during osteogenic differentiation.
Figure 3.5. Activity of a Runx2 reporter during eASC osteogenic differentiation. a Schematic of the Runx2.Hsp68-eGFP lentiviral reporter used to transduce eASCs. The construct contains 343 bp of Runx2 enhancer and Hsp68 minimal promoter upstream to the enhanced green fluorescent protein (eGFP). b Runx2 reporter containing eASCs were transduced with endothelial nitric oxide synthase (eNOS), eNOS+mutated caveolin-1 (eNOS+CAV-1^{F92A}), and eNOS+wild-type caveolin-1 (eNOS+CAV-1^{WT}) following osteogenic induction up to 11 days, and GFP signals were detected by fluorescence microscopy and c increased numbers of osteogenic nodules were observed. *p<0.05, versus eNOS, eNOS+CAV-1^{WT}, eASC (osteogenic induction), and eASC (growth medium). One way ANOVA followed by post hoc. N=3
3.4.7. NO modulates Wnt signaling to promote osteogenic differentiation

To examine the role of canonical and non-canonical Wnt signaling during NO-mediated osteogenic differentiation, expression of Wnt3a, Wnt8a, and Wnt5a was assessed by quantitative real-time PCR. Canonical Wnt ligands Wnt3a (Fig. 3.6a) and Wnt8a (Fig. 3.6b) expressions were up regulated in eASC\textsuperscript{eNOS} and significantly further increased in eASC\textsuperscript{eNOS+CAV-1F92A} (Fig. 3.6a and b, respectively). However, non-canonical Wnt5a expression was reduced in eASC\textsuperscript{eNOS} (Fig. 3.6c), and was significantly further decreased in eASC\textsuperscript{eNOS+CAV-1F92A} (Fig. 3.6c). Treatment with 2 mM L-NAME showed down regulation of Wnt3a expression (Fig. 3.6d) and up regulation of Wnt5a (Fig. 3.6e) in eASC\textsuperscript{eNOS}, indicating that NO modulates Wnt signaling pathway in eASCs.

Furthermore, treatment with NO donor (NONOate) also resulted in increased expression of canonical Wnt ligands Wnt3a (Fig. 3.7a) and Wnt8a (Fig. 3.7b), and down regulation of non-canonical Wnt5a expression (Fig. 3.7c) in a dose-dependent manner from 5 µM to 15 µM of NONOate. Interestingly, when the NO donor concentration was increased up to 20 µM, the effect was completely reversed by down regulating Wnt3a (Fig. 3.7a) and Wnt8a (Fig. 3.7b), and up regulating Wnt5a expression (Fig. 3.7c). Control cells (eASCs in normal growth medium) also showed increased expression of Wnt5a (Fig. 3.7c), suggesting that induction of osteogenic differentiation of eASCs requires activation of canonical Wnt signaling and suppression of non-canonical Wnt5a expression.
To further analyze the relationship between NO-induced osteogenic differentiation and Wnt signaling, eNOS-transduced eASCs were treated with 20 ng/mL of the Wnt signaling inhibitor, Dickkopf-related protein 1 (Dkk-1). Dkk-1 treatment resulted in a significant down regulation of the osteoblast specific markers Alp (Fig. 3.8a) and Runx2 (Fig. 3.8b) compared to untreated eNOS transduced cells.
Figure 3.6. Nitric oxide signaling modulates Wnt signaling in eASCs. Relative mRNA transcript analysis by qPCR shows that endothelial nitric oxide synthase (eNOS) and eNOS+mutated caveolin-1 (eNOS+CAV-1\textsuperscript{F92A}) transduced cells increased the expression of canonical Wnt ligands a Wnt3a and b Wnt8a, whilst downregulating c non-canonical Wnt5a. Relative mRNA transcripts analysis by qPCR shows that treatment with 2 mM L-NAME downregulated d Wnt3a and upregulated e Wnt5a expression. *p<0.05 eNOS+CAV-1\textsuperscript{WT}, CAV-1\textsuperscript{WT}, CAV-1\textsuperscript{F92A}, eASC (osteogenic induction), eASC (growth medium) and #p<0.05 versus eNOS+wild-type caveolin-1 (CAV-1\textsuperscript{WT}), CAV-1\textsuperscript{WT}, CAV-1\textsuperscript{F92A}, eASC (osteogenic induction), and eASC (in growth medium) and ##p<0.05, versus eNOS (L-NAME) eASC (osteogenic induction) and eASC (growth medium). One way ANOVA followed by post hoc. N=3
Figure 3.7. Treatment with nitric oxide donor NONOate modulates Wnt signaling. Relative mRNA transcript analysis by qPCR shows that exogenous NONOate treatment significantly upregulates the expression of canonical Wnt ligands a Wnt3a and b Wnt8a in a dose-dependent manner up to 15 μM with a corresponding downregulation of the non-canonical Wnt ligand c Wnt5a. *p<0.05, versus 0 μM, 5 μM, 10 μM, 20 μM, and 0 μM (growth medium). One way ANOVA followed by post hoc. N=3
Figure 3.8. Inhibition of Wnt signaling through Dickkopf-related protein 1 (Dkk-1) down regulates eASC osteogenic differentiation. Relative mRNA transcript analysis by qPCR shows that Dkk-1 treatment (20 ng/ml) decreased the expression of osteoblast markers a alkaline phosphatase (Alp) and b Runx2 in endothelial nitric oxide synthase (eNOS) transduced eASCs compared to untreated eNOS-transduced eASCs. *p<0.05, versus eNOS (Dkk-1), eASC (osteogenic induction with Dkk-1), eASC (osteogenic induction), and eASC (growth medium). One way ANOVA followed by post hoc. N=3
3.4.8. Canonical Wnt3a promotes osteogenesis while non-canonical Wnt5a suppresses osteogenesis

To explore the opposite effects of canonical and non-canonical Wnt signaling pathways on eASC osteogenesis, canonical Wnt3a and non-canonical Wnt5a expressing lentiviral vectors were generated and transduced eASCs. Wnt3a-transduced eASCs (eASC\textsuperscript{Wnt3a}) and Wnt5a-transduced eASCs (eASC\textsuperscript{Wnt5a}) were incubated in osteogenic induction medium (OM) for 11 days. Interestingly, it was found that, Wnt3a (eASC\textsuperscript{Wnt3a}) promoted osteogenesis as evident by Alizarin Red staining compared to un-transduced eASCs (eASC\textsuperscript{WT}) (Fig. 3.9a). On the other hand, overexpression of Wnt5a (eASC\textsuperscript{Wnt5a}) reduced osteogenic differentiation (Fig. 3.9a). Quantitative analysis of the mRNA levels by real-time PCR revealed that \textit{Alp} (Fig. 3.9b) and \textit{Runx2} (Fig. 3.9c) were up regulated in the eASC\textsuperscript{Wnt3a} culture as compared with the ASC\textsuperscript{WT} culture, and down regulated in eASC\textsuperscript{Wnt5a} culture, suggesting that lentiviral vector-mediated Wnt3a expression can promote osteogenesis while expression of non-canonical Wnt5a suppresses osteogenesis.
Figure 3.9. Lentiviral expression of canonical Wnt3a promotes osteogenesis and non-canonical Wnt5a results in suppressed osteogenesis. a Alizarin Red S staining after 11 days in osteogenic induction medium or growth medium showing that lentiviral Wnt3a transduction increased the calcium deposition, whereas Wnt5a decreased calcium deposition levels. Relative mRNA transcript analysis by qPCR showing that Wnt3a upregulates the expression of osteoblast markers b alkaline phosphatase (Alp) and c Runx2 expression, whereas Wnt5a resulted in downregulation. *p<0.05, versus Wnt5a, eASC (osteogenic induction), and eASC (growth medium). One way ANOVA followed by post hoc. N=3
3.4.9. NO promotes canonical Wnt signaling pathway by promoting nuclear translocation of β-catenin

To further explore mechanisms by which NO promotes canonical Wnt signaling, a lentiviral vector expressing GFP reporter under the control of the Tcf/Lef promoter (TOPFLASH; Addgene) was used. In the canonical Wnt signaling pathway, β-catenin translocation to the nucleus is promoted by the activation of canonical Wnt signaling (Clevers. 2006; Nelson and Nusse, 2004). Accordingly, eASCs were introduced with the lentiviral Tcf/Lef-dGFP reporter, and those cells were then co-transduced with doxycycline inducible eNOS expressing lentiviral vector (Fig. 3.10a). As readout for nuclear translocation of β-catenin, the Tcf/Lef-driven Gfp mRNA expression levels were measured by quantitative real-time PCR. Under osteogenic induction conditions, increased Gfp mRNA expression was demonstrated compared to non-osteogenic induction conditions (cells in growth medium) (Fig. 3.10b). When doxycycline was added to the OM, GFP mRNA expression was significantly increased in the eNOS-transduced cells (eASC eNOS) (Fig. 3.10b). Interestingly, when doxycycline was removed from the medium, Gfp mRNA expression in eASC eNOS was similar to other osteogenic induction conditions (Fig. 3.10b).

Using a β-catenin-specific monoclonal antibody, the effect of NO on β-catenin nuclear translocation was further investigated. eASCs were transduced with the doxycycline inducible eNOS lentiviral vector followed by immunostaining with β-catenin-specific monoclonal antibody (Cell Signaling Technology). When doxycycline was added to the OM, the expression of β-catenin was observed in
eNOS-transduced cells (eASC<sup>eNOS</sup>) (Fig. 3.11a) in both the nucleus and cytoplasm, and when doxycycline was removed from the medium β-catenin expression in eASC<sup>eNOS</sup> was reduced (Fig. 3.11a) to that seen in un-transduced control cells (Fig. 3.11a). Furthermore, nuclear co-localization of β-catenin and DAPI was observed only in doxycycline-treated eASC<sup>eNOS</sup> suggesting that NO may promote nuclear localization of β-catenin (Fig. 3.11b). As a control for the primary antibody, immunostaining was carried out in the absence of the primary antibody specific to β-catenin (Figure 3.S2).

Together, these findings support the paradigm that cellular environments rich in bioavailable NO through either genetic modification or exogenous sources can modulate Wnt signaling, by up regulating the canonical and down regulating the non-canonical pathways resulting in increased osteogenic differentiation (Fig. 3.12).
Figure 3.10. Nitric oxide signaling promotes activity of a β-catenin reporter. **a** Schematic of lentiviral transduction of a β-catenin reporter, TCF/LEF (TOP-dGFP), and a doxycycline (DOX) inducible endothelial nitric oxide synthase (eNOS) transduction and assessment of TCF/LEF activity during osteogenesis. **b** Relative mRNA transcript analysis by qPCR showing eNOS upregulates Gfp mRNA expression when treated with DOX. *p<0.05, versus TOP-dGFP+eNOS (no DOX), TOP-dGFP (osteogenic induction), TOP-dGFP (growth medium), and un-transduced eASC (growth medium). One way ANOVA followed by post hoc. N=3
Figure 3.11. Nitric oxide promotes nuclear translocation of β-catenin. a Immunostaining with a β-catenin-specific monoclonal antibody reveals that the expression of β-catenin in endothelial nitric oxide synthase (eNOS) transduced cells when doxycycline (DOX) is available in the medium. b Nuclear localization of beta catenin in eNOS transduced cells in DOX containing medium. Arrows indicate nuclear localisation of β-catenin.
Figure 3.S2. As a control for specific β-catenin primary antibody binding, immunostaining was carried out in the absence of the primary antibody with no fluorescence signal detected. (a) Detection of nuclear staining by Dapi and (b) fluorescence detection without rabbit monoclonal anti-β-catenin antibody with an anti-mouse IgG secondary antibody conjugated with Alexa 488.
3.5. Discussion

NO plays an important role in osteogenesis, bone remodeling, and metabolism (Evans and Ralston, 1996; Taylor et al. 2006; Wang et al. 2009). It has been reported that both iNOS and eNOS play a role in osteogenesis of embryonic stem cells (Ehnes et al. 2015). Inhibition of iNOS in rat bone marrow derived stem cells has been shown to reduce osteogenic differentiation (Zhang et al. 2015) suggesting that other NO derived from other NOS isoforms also can be attributed to osteogenic differentiation of adults stem cells. We (Bandara et al. 2016) and others (Gomes et al. 2013) have shown that MSCs do not express eNOS. Therefore, in order to investigate the role of eNOS in osteogenic differentiation of eASCs, in this study, eASCs were genetically modified by lentiviral vector-based eNOS. ASCs are promising candidates for stem cell-based therapy for bone repair (Saeed et al. 2016), and the role of eNOS-mediated NO synthesis and its downstream effect on osteogenesis of MSCs remains to be explored. It was found that, eNOS gene transfer by lentiviral vector promoted osteoblast-specific gene expressions (Fig. 3.2e and f), contributing to the matrix mineralization as visualized by Alizarin Red S staining (Fig. 3.2b and d). Noteworthily, this osteogenic potential of eASCs$^{eNOS}$ was significantly abrogated by L-NAME treatment (Fig. 3.3), suggesting that NO derived from eNOS plays a major role in enhancing osteogenesis in eASCs.

CAV-1 is a key negative regulator of eNOS activation and thus inhibits the production of NO (Bernatchez et al. 2005; Bernatchez et al. 2011) and, importantly, CAV-1 is expressed endogenously in MSCs (Baker et al. 2015). The scaffolding
domain (82-101 amino acids) of CAV-1 protein interacts with eNOS at the plasma membrane and this interaction inhibits the eNOS activation reducing NO synthesis (Bernatchez et al. 2005). An alanine scanning approach revealed that substitution of phenylalanine at the amino acid position 92 with alanine to produce CAV-1\textsuperscript{F92A} mutant restored the eNOS activation and promoted NO synthesis (Bernatchez et al. 2005). Thus, in order to understand the contribution of caveolin-1 on the control of NO synthesis in eASC osteogenesis, eASCs were modified by expressing CAV-1\textsuperscript{WT} (as a negative regulator for eNOS activation) or CAV-1\textsuperscript{F92A} (as a positive regulator for eNOS activation) together with eNOS. Confirming a previous observation (Bernatchez et al. 2005), it was found that, co-expression of eNOS and CAV-1\textsuperscript{F92A} increased NO production while eNOS and CAV-1\textsuperscript{WT} co-expressed eASCs showed reduced NO production (Fig. 3.2c), suggesting that CAV-1 is an important regulator of NO production in eASCs. This controlled level of NO synthesis was further found to regulate osteogenesis, where eNOS together with CAV-1\textsuperscript{F92A} resulted in increased osteogenic differentiation of eASCs.

To explore the molecular basis of NO-mediated osteogenesis, the effect of NO on Wnt signaling was investigated. Wnt signaling pathways have been shown to regulate osteoblastogenesis (Cai et al. 2016), in which canonical Wnt ligands promote osteogenesis (Liu et al. 2009; Zhou et al. 2016), and non-canonical Wnt5a can inhibit the canonical Wnt signaling (Topol et al. 2003). In the canonical Wnt pathway, binding of canonical Wnt ligands such as Wnt3a and Wnt8a to cell surface frizzled receptors results in the nuclear translocation of β-catenin (Voloshanenko et al. 2013), which ultimately binds with the Tcf/Lef region to initiate
the transcription of osteogenic genes such as Runx2 (Cai et al. 2016). On the other hand, binding of non-canonical Wnt5a ligand to the Ror2 member of the Ror-family of RTKs inhibits canonical Wnt signaling by promoting β-catenin degradation, and downregulation of β-catenin reduced osteoblast-specific gene expression (Yuan et al. 2011). Results from this study revealed that genetic manipulation of eASCs with eNOS and CAV-1<sup>F92A</sup> (eASC<sup>eNOS+CAV-1F92A</sup>) increased canonical Wnt3a and Wnt8a expression, whereas eASC<sup>eNOS+CAV-1WT</sup> decreased Wnt3a and Wnt8a expression (Fig. 3.6a and b), suggesting that NO levels may regulate Wnt ligand expression and promote osteogenesis. Confirming the role of Wnt signaling on osteogenesis, inhibition of canonical Wnt signaling through Dkk-1 treatment of eNOS expressing cells attenuated osteogenesis as evident by down regulation of osteoblast specific Runx2 and Alp expression (Fig. 3.8). On the other hand, the effect of non-canonical Wnt5a expression was completely the opposite (Fig. 3.6c) to the canonical Wnt3a and Wnt8a expression profiles, suggesting that molecular control of NO synthesis through eNOS/CAV-1 interaction or exogenous NO treatment (Fig. 3.7) results in differential regulation of Wnt ligand expression and their subsequent effect on osteogenic differentiation. Furthermore, it was also found that NO modulates Wnt signaling and promotes osteogenesis when a differentiation environment is enhanced with an optimum concentration of exogenous NO (Figs. 3.4 and 3.7). Interestingly, Neibala et al (2002) have demonstrated that low levels NO (10 µM) can promote T-cell differentiation whilst higher NO levels (>10µM) inhibited T-cell Differentiation.
It has been shown that Wnt3a can directly promote osteogenesis (Cho et al. 2014), whilst Wnt5a plays a role in self-renewal of stem cells (Yeh et al. 2011). It was further investigated the direct effect of canonical Wnt3a and non-canonical Wnt5a on eASC osteogenesis through lentiviral vector overexpression. Interestingly, corroborating previous results on NO-mediated Wnt-regulated osteogenesis, Wnt3a promoted osteogenesis (Fig. 3.9a-c) whereas Wnt5a inhibited osteogenesis (Fig. 3.9a-c). It was shown that increased levels of β-catenin can promote bone formation through increasing the expression of osteoblast-specific genes (Day et al. 2005; Bennett et al. 2005), whilst abnormal osteoblast differentiation has been observed with β-catenin knockdown (Day et al. 2005; Hill et al. 2005). Thus, it is possible that Wnt3a promotes osteogenesis by increasing β-catenin stability and Wnt5a may suppress osteogenesis by degrading β-catenin. NO may regulate this mechanism by increasing Wnt3a and suppressing Wnt5a ligand availability to modulate nuclear localization of β-catenin via the canonical Wnt ligand transduction pathway. In support of this, it was observed that eNOS transduced cells promoted the expression of β-catenin and its nuclear localization (Fig. 3.11), and a Tcf/Lef-dgfp reporter assay demonstrated responsiveness in a NO-rich cellular environment (Fig. 3.10b), which could be controlled through the expression of DOX-inducible eNOS.

3.6. Conclusions

In summary, findings from this study provide an insight into the role of NO in promoting eASC osteogenic differentiation in a cellular environment of optimum
levels of NO through interaction with Wnt signaling pathways. This may lead to the development of novel cell-based therapeutic approaches for bone repair, in particular in vitro modification of MSCs by NO to optimize the endogenous Wnt signaling pathway to promote osteogenic differentiation upon subsequent transplantation.

**Figure 3.12. Proposed signaling mechanism underlying osteogenic differentiation induced by NO in eASCs.** Molecular control of NO levels may activate and suppress the expression of endogenous canonical and non-canonical Wnt ligands, respectively, to promote nuclear localization of β-catenin and subsequent activation of osteogenic differentiation through promoting osteoblast-specific gene transcription.
3.7. References


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Chapter 4: Nitric oxide directed reprogramming of adult rat bone marrow derived mesenchymal stem cells into endothelial like cells via activation of Wnt/β-catenin signalling.
4.1. Abstract

Genetic manipulation of cellular signalling pathways is a novel approach to reprogram adult stem cells towards an endothelial lineage. In this study, rat bone marrow derived mesenchymal stem cells (rBMSCs) were genetically modified to express endothelial nitric oxide synthase (eNOS) and enhance nitric oxide (NO) generation through co-expression of a mutant caveolin-1 (CAV-1\textsuperscript{F92A}). NO plays an important role in maintaining vascular tone and has also been shown to promote stem cell differentiation. eNOS is one of the three enzymes responsible for NO synthesis and is located in membrane caveolae, where it is tightly associated with a scaffolding protein caveolin-1 (CAV-1\textsuperscript{WT}) which inhibits NO production. Firstly rBMSCs were transduced with a lentiviral eNOS-GFP fusion transgene which allowed fluorescence activated cell sorting (FACS) of positive cells. eNOS-GFP expressing rBMSCs (rBMSC\textsuperscript{eNOS-GFP}) increased NO production (6.9 ± 0.8 µM) significantly compared to GFP transduced cells alone (1.04 ± 0.22 µM) and non-transduced control cells (0.91± 0.35 µM) (p<0.05). eNOS-GFP sorted cells were then re-transduced with CAV-1\textsuperscript{F92A} or CAV-1\textsuperscript{WT}, and rBMSC\textsuperscript{eNOS-GFP+CAV-1F92A} showed enhanced in vitro capillary tubule formation and upregulation of endothelial specific CD31 and VE-cadherin gene expression compared to controls. Furthermore to monitor endothelial reprogramming, rBMSC\textsuperscript{eNOS+CAV-1F92A} were transduced with an endothelial specific Flt1 promoter GFP reporter system and showed increased GFP expression compared to controls. Canonical Wnt3a gene expression was increased in rBMSC\textsuperscript{eNOS-GFP+CAV-1F92A} undergoing endothelial differentiation and treatment of rBMSC\textsuperscript{eNOS-GFP+CAV-1F92A} cells with the eNOS
inhibitor L-NAME, resulted in reduced CD31, VE-cadherin and Wnt3a expression. Treatment of rBMSC\textsuperscript{eNOS-GFP+CAV-1F92A} cells with 20 ng/ml of Wnt inhibitor Dkk-1 resulted in decreased CD31 expression suggesting that NO may induce endothelial lineage conversion through canonical Wnt signalling. The relationship between NO and Wnt/β-catenin signalling was further confirmed by western blot analysis of β-catenin expression, in which transduction of eNOS-GFP alone or eNOS-GFP and CAV-1\textsuperscript{F92A} markedly increased β-catenin expression. Finally, subcutaneous implantation of the rBMSC\textsuperscript{eNOS+CAV-1F92A} seeded in polyurethane scaffolds in rats, resulted in formation of blood vessels. Interestingly, rBMSC\textsuperscript{eNOS+CAV-1F92A} demonstrated larger blood vessels formation compared to others. Furthermore NO producing cells significantly reduced histone deacetylase SIRT6 and DNA methyl transferase DNMT1 expression highlighting a role for NO in driving reprogramming events through epigenetic modification. In summary, this study identifies nitric oxide as a potent regulatory molecule which activates Wnt/β-catenin signalling to promote reprogramming of rBMSCs towards an endothelial lineage and may provide a novel platform for vascular regenerative therapy.
4.2. Introduction

Vascular disease is the leading cause of mortality worldwide. According to the world health organization, more than one out of three suffer from vascular related diseases, and it has been estimated that 116 million people will suffer from vascular diseases by 2030. Therefore, a promising area of research is the generation of therapeutic cell types which can be used to generate new blood vessels.

Mesenchymal stem cells (MSCs) have a proliferation capacity and the potential to differentiate into other somatic cell types including endothelial cells (Janeczek Portalska et al. 2012) and smooth muscle cells (Tamama et al. 2008). Unlike embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), MSCs also display immunomodulatory effects upon transplantation into recipients (Glennie et al. 2005; Spaggiari et al. 2006). Thus, MSCs have emerged as a promising cell source for regenerative medicine to treat various disorders such as myocardial infarction (Cai et al. 2016), type 1 diabetes (Katuchova et al. 2015), bone disorders (Kotobuki et al. 2008; Horwitz et al. 2002), and kidney damage (Lee et al. 2006; Choi et al. 2009). This regenerative capacity of MSCs is also associated with their ability to secrete various cytokines such as VEGF, Monocyte Chemoattractant Protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), MIP-1β and monokine induced by IFN-γ (MIG), which are potent in promoting angiogenesis, inhibiting apoptosis and modulating immunoreaction (Boomsma and Geenen, 2012).
Yamanaka and colleagues demonstrated that the expression of four transcription factors (TFs) could reprogram mouse and human cells into a pluripotent stage (Takahashi and Yamanaka, 2006; Takahashi et al. 2007). Subsequent studies have further demonstrated that somatic cell identity can be converted to another by direct trans-differentiation which can be achieved by forced expression of lineage specific TFs, as demonstrated by the successful cell-fate switching of fibroblasts into neuronal, hepatocyte, and cardiomyocyte lineages (Du et al. 2014; Huang et al. 2014; Ieda et al. 2010; Wapinski et al. 2013; Sekiya et al. 2011; Vierbuchen et al. 2010). In addition, it has been demonstrated that small molecules can also be used to reprogram fibroblasts into cardiomyocytes (Cao et al. 2016), and endothelial cells (Lian et al. 2014) by modulating cellular signalling pathways.

In the process of vascular development, multipotent endothelial progenitors (EPCs) initiate angiogenic sprouting to form a mature blood vessel network of arteries and veins and it has been shown that development of endothelial cells (ECs) specific for arteries and venous systems is regulated by intrinsic genetic programs (Aird et al. 2007; Nolan et al. 2013). During the arterial EC development, the Notch signaling pathway directs arterial ECs through activation of a Notch receptor by binding to one of its ligands (Jagged or Delta-like [Dll]) (del Toro et al. 2010; Lawson et al. 2002). The receptor-ligand association followed by a proteolytic cleavage results in the release of the Notch intracellular domain (NICD) into the cytoplasm, followed by translocation into the nucleus where it can activate Hey 1/2 transcription factors which determine arterial EC identity (Lawson et al. 2002; Zhuang et al. 2015). On the other hand, activation of the Notch pathway can
induce arterial specific ephrinB2 and block venous specific EphB4 expression in arterial endothelial cells, whereas an, opposite genetic program is activated in venous ECs through chicken ovalbumin upstream promoter-transcription factor (COUP-TFII) which can mediate venous EC identity (Aranguren et al. 2013; Chen et al. 2012). COUP-TFII is the genetic determinant factor of venous specification by acting upstream of venous specific EphB4 and it can inhibit ephrinB2 (You et al. 2005; Swift et al. 2009; Chen et al. 2012). It has been shown that COUP-TFII in arterial endothelial cells can inhibit arterial specific markers, Nrp1 and Jagged1 (You et al. 2005) and also can inhibit Notch1 and Hey1/2 during the venous EC development (Aranguren et al. 2013; Chen et al. 2012). COUP-TFII is also important for lymphatic EC development where it interacts with Prox1 transcription factor to initiate lymphatic EC development, indicating that arterial-venous determination is followed by lymphatic specification during embryonic development (Yamazaki et al. 2009; Srinivasan et al. 2010). The lymphatic vascular system originates from venous endothelial cells (Srinivasan et al. 2010), in which, a subpopulation of venous ECs starts to express Prox1 and Sox18 (Adams and Alitalo, 2007; Kiefer and Adams, 2008) to initiate the lymphatic differentiation. Previous evidence has shown that Prox1 is a master regulator of lymphatic EC identity as Prox1 mutant mice fail to develop lymphatic ECs and do not express lymphatic markers such as VEGFR3 and LYVE1 (Wigle and Oliver, 1999; Wigle et al. 2002) whilst Sox18 knockout mice have not shown lymphatic defects which may be due to genetic compensation by Sox7 and Sox17 (Hosking et al. 2009).
Nitric oxide (NO) is a signalling molecule with a short half-life (Ignarro et al. 1987; Vaughn et al. 1998; Lancaster, 1997), is synthesized from L-arginine by nitric oxide synthases one of which is eNOS (Thomas, 2015). eNOS is localized mainly in specific intracellular membrane domains, including the Golgi apparatus (Iwakiri et al. 2006) and plasma membrane caveolae through interaction with caveolin-1 (Bernatchez et al., 2005). A previously demonstrated direct interaction of eNOS with wild-type caveolin-1 (CAV-1WT) (Bernatchez et al. 2005) has been shown to inhibit eNOS activation and has been demonstrated that, a mutated version of CAV-1 with a phenylalanine to alanine substitution at the amino acid position 92 (CAV-1F92A) resulted in increased NO production (Bernatchez et al. 2005). NO controls variety of physiological effects such as regulating blood pressure via smooth muscle relaxation (Tare et al. 1990), controlling cell proliferation (Davis et al. 2001), modulating apoptosis (Vaughn et al. 1998), promoting growth factor-induced angiogenesis (Lancaster, 1997), and accelerating wound healing (Schanuel et al. 2015). It has been shown that, NO drives chemical modification of proteins through S-nitrosylation to mediate these responses (Hess et al. 2005) or through epigenetic modification (Hickok et al. 2013). Bernatchez et al have demonstrated that, delivery of F92A mutated CAV-1 peptides have been shown to increase eNOS derived NO synthesis and promote vascularization in mice (Bernatchez et al. 2011). Ha and colleagues have demonstrated that, blood vessels formation in the retina was attenuated in eNOS knockout mice (Ha et al. 2016). The importance of eNOS for arterial vessels has been shown through impairment of artery development in eNOS-deficient mice (Rennie et al. 2015).
Furthermore, Yang et al demonstrated that inhibition of Jak2, an upstream component of eNOS, attenuates endothelial function due to repression of eNOS (Yang et al. 2013). Finally, endothelial specific Gab1 knockout mice display impaired angiogenesis and interestingly biochemical analysis has identified a marked decrease in eNOS activation in Gab1 knockout mice suggesting that impaired angiogenesis is due to eNOS inactivation (Lu et al. 2011).

Several studies have described the importance of canonical Wnt signalling in mesoderm commitment during embryogenesis (Tam and Loebel, 2007). For example, mice with impaired Wnt signalling were unable to develop mesoderm (Huelsken et al. 2000). Canonical Wnt signalling has been shown to induce human pluripotent stem cell (hPSC) differentiation towards mesoderm (Woll et al. 2008), cardiomyocytes (Brade et al. 2006, Lian et al. 2012) and endothelial cells (Sumi et al. 2008, Lian et al. 2014) indicating that Wnt/β-catenin signalling is a key pathway which can determine vascular lineage specification. Pharmacological activation of the canonical Wnt pathway through lithium has been shown to promote retinal vascular development in mice, in which treatment of Lrp5 knockout mice with lithium was able to restore canonical Wnt signalling and promote vascular development (Wang et al. 2016). The importance of Wnt/β-catenin signalling has further been demonstrated with human mesenchymal stem cells, in which β-catenin deleted MSCs could not differentiate into endothelial cells in vitro and showed impaired vasculogenesis in vivo (Zhang et al. 2016). Finally, tumorigenicity 15 protein (ST15) also known as Reck has been shown to induce cerebral vasculature by promoting canonical Wnt signalling, in which Reck knockout mice
showed impaired cerebral vascular development due to down regulation of the canonical Wnt pathway (Ulrich et al. 2016). Together, in this study the hypothesis that molecular control of NO synthesis in rat bone marrow derived mesenchymal stem cells (rBMSCs) can promote endothelial differentiation was tested. To regulate eNOS-NO signalling, eNOS-CAV-1WT and eNOS-CAV-1F92A interactions were created in rBMSCs. The results indicate that the increased level of NO induces endothelial differentiation specifically towards arterial endothelial cell subtype through activation of the downstream Wnt/β-catenin signalling pathway.
4.3. Materials and methods

4.3.1. Cell culture

Rat bone marrow derived mesenchymal stem cells (rBMSCs) were isolated from bone marrow as described in chapter 2. All sampling was carried out using protocols approved by the St. Vincent’s hospital Animal Care and Ethics Committee. Human embryonic kidney 293T cells (HEK293T) (ATCC, Manassas, VA) and rBMSCs were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10 % (v/v) fetal bovine serum (Lonza), 100 U/ml penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine (Growth medium) at 37ºC and 5 % CO₂, and maintained.

4.3.2. Plasmid constructs

The list of lentiviral plasmids used in this study is listed in table 4.1, which were used for construction of lentiviral vectors. HF Phusion (NEB) DNA polymerase was used for all the polymerase chain reactions (PCRs) and all the restriction endonucleases were purchased from New England Biolabs (NEB) unless indicated otherwise.
Table 4.1. Lentiviral vectors and reporter constructs used in this study

<table>
<thead>
<tr>
<th>Lentiviral vector</th>
<th>Relevant properties (Promoters)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLVX-eNOS-GFP</td>
<td>CMV-eNOS-eGFP</td>
<td>This study</td>
</tr>
<tr>
<td>pWPT-eNOS</td>
<td>CMV-eNOS</td>
<td>This study</td>
</tr>
<tr>
<td>pWPT-CAV-1F92A</td>
<td>CMV-CAV-1F92A</td>
<td>This study</td>
</tr>
<tr>
<td>pLVX-CAV-1WT</td>
<td>CMV-CAV-1WT</td>
<td>This study</td>
</tr>
<tr>
<td>pTRIP-Flt1-eGFP</td>
<td>Flt1-eGFP</td>
<td>This study</td>
</tr>
</tbody>
</table>

To generate an eNOS-GFP fusion expression lentiviral vector, a codon optimized cDNA of human eNOS was amplified using forward (5'/-ATCAGAATTTCATGGGCAACCTGAA-3'/) and reverse (5'//-ATCAGGATCCCCATCAGGGGCTGT-3'/) primers and introducing EcoR1 and BamH1 restriction sites (underlined sequences in both the primers) omitting the stop codon. The resulted stop codon lacking eNOS cDNA was sub-cloned into the lentiviral plasmid pLVX-GFP (Clontech, Mountain View, CA) at the EcoR1 and BamH1 restriction sites in the multiple cloning sites downstream to the CMV promoter obtaining a GFP fusion. The cloning was confirmed by double digestion of the resulted plasmid encoding eNOS with EcoR1 and BamH1 yielding a fragment of ~3.7 kb (Fig. 4.1a). Human wild type cavolin-1 (CAV-1WT) expressing lentiviral vector and the mutated caveolin-1 (CAV-1F92A), in which phenylalanine (F) at the amino acid position 92 was replaced into alanine (A) expressing lentiviral vector construction was described in chapter 3.
4.3.3. Reporters

A 1049-bp fragment of the endothelial specific Flt1 promoter region was synthesized (GenScript, NJ, USA). The entire fragment was then subcloned into pTRIP-eGFP lentiviral vector at the MluI and BamH1 restriction sites replacing a rat insulin promoter (RIP), upstream of the enhanced GFP (eGFP) coding sequence (full sequence has been provided in the appendix 4).

4.3.4. Lentiviral vector production and transduction of rBMSCs

Lentiviral vectors used in this study (Table 4.1) were generated by four plasmid transfection of HEK293T cells. Briefly, each well of a six-well tissue culture plate was coated with 50 μg/ml of DL-lysine (Sigma-Aldrich) in phosphate-buffered saline (PBS) and incubated for 2 h at 37 °C. HEK293T cells were then seeded at a density of 1 × 10^6 cells per well, 24 h prior to transfection of 6.3 μg of packaging plasmid psPAX2 (Addgene), 3.1 μg of Rev expression plasmid pRSV Rev (Addgene), 3.5 μg of VSV-G envelope expression plasmid pMD2.G (Addgene), and 10 μg of the gene of interest expression transfer vector using a standard calcium phosphate transfection method (Tiscornia et al. 2006). Seventeen hours post-transfection, the media was changed and supernatant containing lentiviral particles were collected at 48 h and 72 h post-transfection, combined, and filtered through a 0.45-μM PVDF filter, and used for rBMSC transduction in the presence of 4 μg/ml Polybrene (Sigma-Aldrich).
4.3.5. Fluorescence-activated cell sorting (FACS) and culture expansion

rBMSCs were transduced with lentiviral eNOS-GFP or GFP and 72 h of post transduction, the cells were trypsinised, pelleted and resuspended in FACS buffer (PBS containing 5% FBS and 10 μM rock inhibitor Y-27632). The cells were then analysed and GFP+ cells were sorted using a FACSAlia cell sorter (BD Biosciences). GFP negative gate was set based on florescence minus un-transduced rBMSCs. Sorted GFP+ cells were collected directly into FACS tubes containing growth medium and 10 μM rock inhibitor Y-27632 followed by replating in a 6-well tissue culture plate. After 24 h, the medium was replaced with growth medium and incubated for 6 days with medium changed every 2-3 days to obtain enough cells for downstream experiments.

4.3.6. Endothelial reprogramming

To investigate the effect of eNOS-NO signalling on endothelial reprogramming, lentiviral-transduced rBMSCs and non-transduced cells were seeded in duplicate into a 12-well polystyrene tissue culture plate at 30,000 cells / well, and incubated for 24 h, followed by a second transduction with CAV-1WT, and CAV-1F92A (Fig 4.3). The transduced cells were then incubated in growth medium for a further 72 h with one medium change and then cells were harvested and analysed for gene, and protein expression, in vitro tubule formation assay and in vivo transplantation (Fig 4.3).
4.3.7. *In vitro* tubule formation assay

*In vitro* capillary formation was performed as described previously (Faulkner et al. 2014 and chapter 6). Briefly, Geltrex™ (Life technologies) was thawed on ice overnight and applied evenly over each well (50 μl) of a 96-well plate and incubated for 30 min at 37°C allowing polymerisation. Lentiviral vector transduced rBMSCs or control cells were seeded at 12,000 cells per well and grown in 100 μl angiogenic induction medium (DMEM (Sigma-Aldrich), 1.5 % FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine and incubated at 37°C for 4 h to allow capillary network formation and cells were then fixed with 4 % paraformaldehyde.

4.3.8. Quantitative real time PCR (RT-qPCR)

Total RNA from lentiviral transduced and control cells after 3 days in culture was isolated using TriReagent (Thermo Fisher Scientific) according to the manufacturer’s instructions and the concentration of isolated RNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific). RNA was treated with RQ1 RNase free DNase (1 U/1 μg RNA; Promega, WI, USA). cDNA was synthesized with 1 μg RNA from all samples using a High Capacity Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative real-time PCR assays were performed on a QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific) using the SsoFast EvaGreen Supermix (Bio-Rad). Primer sequences used for target gene amplification are described in Table 4.2. Assays
were performed in duplicate and target gene expression was normalized to rat GAPDH mRNA levels using the \( \Delta\Delta C_t \) method.

**Table 4.2. Primers used for RT-qPCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5' &gt; 3')</th>
<th>Reverse (5' &gt; 3')</th>
<th>Accession number</th>
</tr>
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<td>CD31</td>
<td>CATTGGTTACCTCGGGAGTC</td>
<td>GTCTTCACCAGCCTTTCTC</td>
<td>NM_001107202</td>
</tr>
<tr>
<td>VE-cadherin (cadherin 5)</td>
<td>CAGGGCAAGCTGGTAGTACA</td>
<td>TGAAGTTGCTGCTCCTGTC</td>
<td>NM_001107407</td>
</tr>
<tr>
<td>Notch1</td>
<td>GAACCTCGGGATCATGGATTAG</td>
<td>ACCTGGTACTGGGTAAGCA</td>
<td>NM_001105721</td>
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<tr>
<td>Dil4</td>
<td>CTCTCTGTGGGATCTGGATTT</td>
<td>CAAGTCTCTCCTCTGCTTC</td>
<td>NM_001107760</td>
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<td>Hey2</td>
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<tr>
<td>Notch 4</td>
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<tr>
<td>EphrinB2</td>
<td>GTTCCGAGTTGGGCTTATT</td>
<td>GTGTGCTGGAGAGGTGTTT</td>
<td>NM_001107328</td>
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<tr>
<td>Coup-tll (nr212)</td>
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<td>GCTGCCGGACAGTAACATA</td>
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<tr>
<td>EphB4</td>
<td>GGAACAGGACGGGCTTATAG</td>
<td>GAGGAAGAGGAAGGACTA</td>
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<td>Lefty 1</td>
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<td>Lefty 2</td>
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<td>Prox1</td>
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<tr>
<td>Sox18</td>
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<td>CCCAGAAGAGGGTGGTGAATG</td>
<td>NM_001024781.1</td>
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<tr>
<td>Lyve1</td>
<td>CAAGGAGGTCTGTAAGGTTCTG</td>
<td>GTTCTGCAAGCCATCATCATAG</td>
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<tr>
<td>Dnmt1</td>
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<td>GGCCTACTCTGCTCTGGTT</td>
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<td>Dnmt3a</td>
<td>GCCCATGCTCTCGTGGATT</td>
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<td>NM_001003958</td>
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<tr>
<td>Dnmt3b</td>
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<td>AGTACTGAAAGCAGCAGAGAG</td>
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<tr>
<td>Sirt6</td>
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<td>CTTCACATACCTCCTCAGCA</td>
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<tr>
<td>Gapdh</td>
<td>ACAGCAACAGGGTGTTGGGAC</td>
<td>TTGGAGGGTGCGCAACTT</td>
<td>NM_017008.4</td>
</tr>
</tbody>
</table>
4.3.9. Western blot analysis

Lentiviral transduced (MOI 10) and un-transduced control cells were washed with ice-cold PBS (Sigma-Aldrich) twice, and lysates were prepared by homogenization in RIPA buffer (Sigma-Aldrich), following mixing with 4 × NuPAGE LDS sample buffer (Life technologies) and lysed by heating for 10 min at 70°C. Total proteins were separated by 4–12 % Bis-Tris NuPAGE (Novex, Life technologies) and transferred to a PVDF membrane (Millipore). After blocking with odyssey blocking buffer (LI-COR) for 30 min at room temperature, the membrane was incubated with primary antibodies specific to eNOS (1:1000 dilution, Abcam), p-eNOS (1:1000, Cell signalling technology), CAV-1 (1:1000, Cell signalling technology), Akt (1:1000, Cell signalling technology), β-catenin (1:1000, Cell signalling technology) and β-actin (1:1000, LI-COR) overnight at 4°C. The membrane was washed with 0.1 % tween in PBS three times for 10 min each, incubated with donkey anti-rabbit IgG (H&L) (Alexa Fluor® 680) or goat anti-mouse donkey anti-rabbit IgG (H&L) (Alexa Fluor® 680) secondary antibodies (1:20,000, Life technologies) at room temperature for 1 h, and antibody-bound proteins were visualized by fluorescence detection with a LI-COR odyssey system.

4.3.10. Reporter assays

For the GFP based reporter assays using the endothelial promoter, FLT1 driven GFP expression, the cells were transduced with the FLT1-eGFP lentivector and were then subjected to fluorescence based microscopic analysis.
4.3.11. Nitrite levels detection

Extracellular nitrite level was measured using the Griess reagent (Promega) according to the manufacturer’s instructions and measurement of absorbance at 540 nm. Triplicates of each sample were measured. (Standard curve (from 0.1 µM to 10 µM) was prepared).

4.3.12. In vivo implantation

Male nude rats were purchased from Animal Resources Centre (Perth, Australia) and maintained with a 12 hours light/dark cycle and given water and food. Experimental procedures were approved by St. Vincent’s Hospital (Melbourne) Animal Ethics Committee.

4.3.13. Aseptic technique

To maintain sterile conditions, all surgical equipment was autoclaved at 125°C for 15 min. Animals were given Baytril as antibiotic in drinking water (0.175 mg/mL) for three days before and after surgery.

4.3.14. Surgery and cell transplantation

After the animal was anesthetized, the animal was placed on the operating table. A longitudinal incision was made along the dorsal skin and surgically exposed. The
polyurethane scaffolds containing cells (Further details in section 2.6, chapter 2) were then placed subcutaneously with four scaffolds located along each left and right side. The scaffolds were attached internally with silk sutures followed by skin closure with sutures. Animals were treated post-operatively with analgesic Carprofen (Pfizer, NY, USA) at 5 mg/kg given subcutaneously and recovered from anaesthesia. The animals were then housed for 7 days after which, the animals were euthanized and cell-scaffold implants were removed and fixed in 4% paraformaldehyde for 4 h and processed as described below.

4.3.15. Histological analysis

4.3.15.1. Tissue fixation, processing, and paraffin embedding

Tissues were fixed in 4% paraformaldehyde (in PBS, pH 7.4) for 4 h at room temperature. To maintain the shape of tissue, after fixation the tissue was embedded in 1.5% agarose containing formalin (1.5 g agarose, 10 ml 37% formaldehyde, and 90 ml distilled water), following, fixation in 4% paraformaldehyde at 4°C overnight. The tissues were processed for 24 h using an automated tissue processor (Shandon Hypercenter XP, ThermoShandon, MA, USA), where the tissues were dehydrated through serial changes of alcohol, cleared in histolene, and infiltrated with paraffin. Tissues were then divided into equal transverse slices and embedded in paraffin. Paraffin blocks were sectioned at 5 µm using a microtome (1130/Biocut Reichert-Jung microtome, Leica Microsystems, AG, Germany). Sections were placed in a 45°C water bath and
mounted onto microscope slides, dried overnight at 37°C and stored at room temperature.

4.3.15.2. Immunohistochemistry

Paraffin embedded sections (5 μm) were stained with eosin-hematoxylin (H&E) to visualise general tissue morphology and the detailed protocol is described in the General methods chapter (Chapter 2, Section 2.6.5). To visualise GFP expression in transplanted cells, scaffold-sections were subjected to heat-mediated antigen retrieval in citric acid buffer (pH 6.0, 30 minutes at 95°C) and this was followed by quenching with 3% H₂O₂ for 5 minutes. The sections were then incubated in serum-free blocking solution (Ultra-V block, Thermo Fisher Scientific, MA, USA) for 5 minutes followed by incubation with anti-GFP antibody (1:200, Santa Cruz) for overnight at 4°C. The sections were then incubated with biotinylated rabbit-anti-mouse secondary antibody for 60 minutes and avidin-biotinylated-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, UK) for 30 minutes followed by visualisation with diaminobenzidine (DAB) chromogen (Thermo Fisher Scientific) and sections were then counterstained with hematoxylin and dehydrated through a concentration series of ethanol to histolene (Chapter 2, Section 2.6.5) and mounted with DPX mounting medium and cover slipped.

To visualise CD31 expression in transplanted cells in rat, endogenous peroxidase activity of sections was quenched with 3% H₂O₂ for 10 minutes followed by enzymatic-mediated antigen retrieval with Proteinase K (Dako, Hamburg, Germany) for 5 minutes. Sections were then blocked in serum-free blocking
solution (Ultra-V block, Thermo Fisher Scientific) for 5 minutes, and then incubated with anti-mouse CD31 (1:100, Abcam) for overnight at 4°C. Sections were then incubated with biotinylated rabbit-anti-mouse secondary antibody for 60 minutes, and avidin-biotinylated-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, UK) for 30 minutes followed by visualisation with diaminobenzidine (DAB) chromogen (Thermo Fisher Scientific) and sections were then counterstained with hematoxylin and dehydrated through a concentration series of ethanol to histolene (Chapter 2, Section 2.6.5) and mounted with DPX mounting medium and cover slipped.

To identify blood vessels in transplanted scaffold constructs, endogenous peroxidase activity of sections was quenched with 3% H₂O₂ for 10 minutes followed by enzymatic-mediated antigen retrieval with Proteinase K (Dako, Hamburg, Germany) for 5 minutes. Sections were then blocked in serum-free blocking solution (Ultra-V block, Thermo Fisher Scientific) for 5 minutes, and then incubated with biotinylated Griffonia Simplicifolia lectin (Vector Laboratories) overnight at 4°C and this was followed by HRP-streptavidin (Dako) treatment for 30 minutes. The sections were then counterstained with hematoxylin. The vascular volume was calculated by the following equation. % of vascular volume = No of vessels/ no of vessels + total tissue x 100.
4.3.16. Statistics

Data are presented as mean ± SEM. The statistical significance of differences between two groups was determined by Student’s t test. Statistical analysis for nitric oxide levels, RT-qPCR data was performed using one way analysis of variance (ANOVA) followed by post hoc. All tests were performed using the statistical software Graphad Prism 6 (GraphPad, LaJolla, CA, USA). p<0.05 was considered statistically significant.
4.4. Results

4.4.1. Construction and validation of a GFP fused eNOS lentiviral vector

First, the successful construction of eNOS-GFP fusion expression lentiviral vector, was confirmed by double digestion of the lentiviral plasmid encoding eNOS with EcoR1 and BamH1 yielding a fragment of ~3.7 kb (Fig 4.1a).

Next, lenti eNOS-GFP construct was used to generate lentiviral vectors which were used to transduce rBMSCs to confirm protein expression and validation of functionality. Protein expression was assessed by western blot analysis (Fig 4.1b) and immunostaining (Fig 4.1c) conforming the expression of eNOS protein only in eNOS delivered cells but not in GFP delivered or untransduced cells (Fig 4.1b and 1c). Finally, nitrite levels was assessed by Griess assay resulting increased level of NO production in eNOS-GFP delivered cells compared to GFP transduced and untransduced cells (Fig 4.1d).
**Figure 4.1. Functional characterizations of eNOS-GFP construct.** (a), eNOS cDNA sequence was PCR amplified and subcloned into the pLVX-eGFP plasmid and ligation was confirmed by double digestion. (b), Lentiviral vectors expressing eNOS-GFP or GFP (negative control) were used to transduce rBMSCs and the expression of eNOS was confirmed by western blot analysis and (c), immunofluorescence microscopy with eNOS specific antibody or visualization of GFP fluorescence. (d), significant increase of nitrite level was seen in lenti (Lv)-eNOS-GFP transduced rBMSCs as measured by Griess assay. *p<0.05 vs. compared with lenti (Lv)-GFP transduced cells and untrasduced rBMSCs. One way ANOVA followed by post hoc.
4.4.2. A gene modified rBMSC system to study endothelial differentiation

To select a homogenous population of lentiviral eNOS transduced cells, a C-terminal GFP fused eNOS coding region in a lentiviral vector background was designed and used to transduce to rBMSCs. GFP fluorescing cells were then selected by fluorescence activated cell sorting (FACS) of eNOS-expressing cells (Fig 4.2b) (eNOS-GFP expressing rBMSCs designated as rBMSC\textsuperscript{eNOS-GFP}). As a control a GFP expressing lentiviral vector was used to transduce rBMSCs followed by FACS analysis (Fig 4.2a). The sorted GFP (Fig 4.2d, rBMSC\textsuperscript{GFP}) and eNOS-GFP (Fig 4.2e, rBMSC\textsuperscript{eNOS-GFP}) cells were replated and observed after 24 h by fluorescence microscopy. Un-transduced cells were used as a negative control for GFP expression (Fig 4.2c; rBMSC\textsuperscript{WT}).
Figure 4.2. Fluorescence activated cell sorting (FACS) analysis for lentiviral vector transduced rBMSCs. rBMSCs were transduced with GFP or eNOS-GFP expressing lentiviruses and GFP positive (GFP+) cell populations were sorted by FACS. (a), GFP lentivirus and (b), eNOS-GFP lentivirus transduced rBMSCs showed approximately 72% and 11% GFP+ cells respectively, and (c), un-transduced rBMSCs showed no GFP expression. Following FACS, GFP expression in (d), GFP and (e), eNOS-GFP transduced cells were evaluated by fluorescence microscopy.
4.4.3. eNOS gene transfer promotes eNOS-NO signalling in rBMSCs

The NO signalling pathway in the eNOS expressing cells was analysed through western blot analysis of protein expression together with treatment of cells with inhibitors of NO production. First, the sorted rBMSC^{eNOS-GFP} and rBMSC^{GFP} cells were grown for 6 days in growth medium and then transduced separately with lentiviral vectors expressing either CAV-1^{WT} or CAV-1^{F92A}, a schematic of the experimental plan is shown in figure 4.3. The cells were then incubated for another 72 h and analysis of proteins involved in NO signalling was performed. Firstly, the expression of eNOS was only evident in rBMSC^{eNOS-GFP} cells and was not affected by co-transduction with either CAV-1^{WT} or CAV-1^{F92A} (Fig 4.4a). Cells expressing only CAV-1^{WT} or CAV-1^{F92A} did not show eNOS expression (Fig 4.4a), whilst overexpression of CAV-1^{WT} or CAV-1^{F92A} alone or with eNOS markedly increase CAV-1 expression (Fig 4.4b). Treatment with 2mM L-NAME (NOS inhibitor) resulted in reduced eNOS expression in rBMSC^{eNOS-GFP+CAV-1^{F92A}} cells (Fig 4.4a). Interestingly, phosphorylation of eNOS at the serine^{1179} residue was markedly increased with eNOS-GFP+CAV-1^{F92A} co-transduction and, decreased with eNOS-GFP+CAV-1^{WT} co-transduction and L-NAME treatment (Fig 4.4a). NO production as measured by nitrite accumulation using the griess assay (Fig 4.4c) was increased in the eNOS-GFP and eNOS-GFP+CAV-1^{F92A} transduced cells and was reduced with L-NAME treatment. NO production would appear to correlate with the phosphorylation status of eNOS (Fig 4.4a). Furthermore, the expression of protein kinase B (AKT) which is a downstream target of NO was markedly increased in eNOS-GFP and CAV-1^{F92A} co-transduced cells (rBMSC^{eNOS-GFP+CAV-}
1F92A) again demonstrating a relationship with enhanced phosphorylation of eNOS (Fig 4.4a). In addition, the western blot analysis demonstrated that the expression of caveolin-1 in all the cells demonstrating both endogenous expression and increased expression resulting from transgene overexpression in cells transduced with either CAV-1WT or CAV-1F92A (Fig 4.4a).

**Figure 4.3. Schematic of lentiviral transduction and endothelial reprogramming strategy.**
rBMSCs were transduced with eNOS-GFP and GFP separately and 72 h post transduction, GFP expressing cells were sorted and re-plated. Following a further 6 days incubation in growth medium, the eNOS-GFP and the GFP cells were re-transduced with lentiviral vectors expressing CAV-1WT or CAV-1F92A and incubated for a further 72 h (with or without 2 mM L-NAME) and then were subjected to downstream analysis.
Figure 4. Expression of key proteins in the NO signalling pathway in transduced rBMSCs and control cells. (a), Western blot analysis of expression of AKT, eNOS, phosphorylated eNOS and caveolin-1 and (b), Quantification of CAV-1 protein expression. (c), NO production in transduced cells and controls as measured by griess assay. *p<0.05 and **p<0.05 vs. eNOS-GFP+CAV-1F92A (L-NAME), eNOS-GFP+CAV-1WT, CAV-1WT, CAV-1F92A, GFP, and rBMSCs. ##p<0.05 vs rBMSCs, GFP, and eNOS. (n=3). One way ANOVA followed by post hoc
4.4.4. Nitric oxide promotes *in vitro* capillary formation

The ability of the eNOS-GFP and CAV-1$^{F92A}$ lentiviral transduced cells to produce *in vitro* capillary like structures was assessed. Transduced cells were plated on a 96-well cell culture plate coated with extracellular matrix (Geltrex™) and incubated for 4 h. The rBMSC$^{eNOS-GFP+CAV-1F92A}$ and rBMSC$^{eNOS-GFP}$ transduced cells only formed capillary like structures compared to the other transduced cells and controls (Fig 4.5a), in which eNOS+CAV-1$^{F92A}$ (17.21 ± 0.55 mm) and eNOS (14.24 ± 0.56 mm) transduced rBMSCs formed significantly longer network compared to other treatments. This *in vitro* capillary formation was significantly impaired by rBMSC$^{eNOS-GFP+CAV-1WT}$ cells suggesting over-expression of CAV-1$^{WT}$ inhibits NO production and specific inhibition of eNOS activity with 2 mM L-NAME in co-transduced rBMSC$^{eNOS-GFP+CAV-1F92A}$ cells also inhibited capillary formation.

4.4.5. eNOS promotes rBMSCs commitment towards an endothelial lineage.

To assess whether the eNOS-GFP fusion construct could induce endothelial specific gene expression, we assessed endothelial specific *Cd31* and *Ve-cadherin* gene expression by quantitative real time PCR. A significant increase in *Cd31* (Fig 4.5b) and *Ve-cadherin* (Fig 4.5c) expression in rBMSC$^{eNOS}$ transduced cells were observed compared to rBMSC$^{GFP}$, and un-transduced cells. Compared with eNOS-GFP transduced cells alone, *Cd31* (Fig 4.5b) and *Ve-cadherin* (Fig 4.5c) mRNA expression was significantly increased in the co-transduced rBMSC$^{eNOS-GFP+CAV-1F92A}$ cells, but not in rBMSC$^{eNOS-GFP+CAV-1WT}$ demonstrating that increased levels of NO due to the CAV-1$^{F92A}$ protein can promote eNOS mediated *Cd31* and *Ve-
cadherin expression whilst CAV-1WT has an inhibitory effect. The role of NO in mediating Cd31 and Ve-cadherin expression was shown by treatment of rBMSCeNOS-GFP+CAV-1F92A with 2 mM L-NAME which resulted in reduced Cd31 (Fig 4.5b) and Ve-cadherin (Fig 4.5c) expression.

Figure 4.5. Nitric oxide promotes endothelial differentiation of rBMSCs. (a), Capillary network formation by lentiviral transduced and non-transduced control cells. Relative mRNA expression of endothelial-specific (b), Cd31 and (c) Ve-cadherin in eNOS-GFP and eNOS-GFP+CAV-1F92A transduced rBMSCs as assessed by quantitative real time PCR. *p<0.05 and †p<0.05 vs. eNOS-GFP+CAV-1F92A (L-NAME), eNOS-GFP+CAV-1WT, CAV-1WT, CAV-1F92A, GFP and un-transduced rBMSC. Scale bars=50 µm. (n=3). One way ANOVA followed by post hoc.
4.4.6. Activity of a Flt1 reporter and Flt1 gene expression in transduced rBMSCs

To further validate induction of endothelial differentiation by NO, an endothelial reporter system was developed, which is specifically activated in endothelial lineage committed cells. This reporter system consists of the Flt1 promoter upstream to the enhanced green fluorescence protein (eGfp) coding sequence driving the expression of Gfp (Fig 4.6a). To validate promoter specificity, a lentiviral vector expressing the Flt1-eGfp was used to transduce human microvascular endothelial cells (HMECs) (Fig 4.6b) and non-endothelial rBMSCs (Fig 4.6c). After 5 days in growth medium only transduced HMECs were positive for Gfp expression (Fig 4.6b) compared to the rBMSCs demonstrating that insertion of this reporter construct into the rBMSC genomic locus can faithfully report the endothelial lineage conversion. Flt1-eGfp reporter transduced rBMSCs were then co-transduced with lentiviral vectors expressing eNOS (without Gfp fusion; pWPT-eNOS, Table 4.1), eNOS+CAV-1WT, eNOS+CAV-1F92A, CAV-1WT, and CAV-1F92A. After 5 days post transduction, GFP expression was only seen in eNOS and, eNOS+CAV-1F92A transduced cells and which also appeared higher in eNOS+CAV-1F92A transduced cells (Fig 4.6c). In addition to the Flt1-eGfp reporter expression data, gene expression analysis also confirmed the induction of endogenous Flt1 gene expression in rBMSCeNOS+CAV-1F92A and rBMSCeNOS (Fig 4.6d) and this was significantly reduced in rBMSCeNOS+CAV-1WT and also in 2mML-NAME treated rBMSCeNOS+CAV-1F92A (Fig 4.6d).
Figure 4.6. Activity of an Flt1 reporter during genetically engineered rBMSCs differentiation. (a) Schematic of the Flt1-eGFP lentiviral reporter used to transduce rBMSCs. The construct contains 1049-bp of the human Flt1 promoter upstream to the enhanced green fluorescent protein (eGFP). (b) Flt1 reporter was validated by transducing human microvascular endothelial cells (HMECs). (c), Flt1 reporter containing rBMSCs were re-transduced with lentiviral vector expressing endothelial nitric oxide synthase (eNOS), eNOS+mutated caveolin-1 (eNOS+CAV-1^F92A), and eNOS+wild-type caveolin-1 (eNOS+CAV-1^WT) following incubation in growth medium up to 5 days, and GFP signals were detected by fluorescence microscopy. (d), RT-qPCR analysis for Flt1 mRNA expression in transduced cells. *p<0.05 and #p<0.05 vs. eNOS+CAV-1^F92A (L-NAME), eNOS+CAV-1^WT, CAV-1^WT, CAV-1^F92A, GFP and rBMSC. (n=3). One way ANOVA followed by post hoc
4.4.7. Nitric oxide promotes angiogenic responsive gene expression.

Gene expression of angiogenic markers was assessed in lentiviral transduced cells. Expression of Vegf-a (Fig 4.7a), and Fgf2 (Fig 4.7b) and their corresponding receptors Pdgfra (Fig 4.7c) and Fgfr2 (Fig 4.7e) were increased in rBMSC<sup>eNOS-GFP</sup> cells and expression was further increased in rBMSC<sup>eNOS-GFP+CAV-1F92A</sup> cells suggesting that increased levels of eNOS mediated NO may promote angiogenic responsive gene expression. Supporting this hypothesis, these gene expression profiles were abrogated in rBMSC<sup>eNOS-GFP+CAV-1WT</sup> and in rBMSC<sup>eNOS-GFP+CAV-1F92A</sup> treated with 2mM L-NAME (Fig 4.7a-e). In addition, induction of Icam-1 (Fig 4.7d) and Kdr/Vegfr2 (Fig 4.7e) gene expression was also increased in rBMSC<sup>eNOS-GFP</sup> and they were further increased in rBMSC<sup>eNOS-GFP+CAV-92A</sup> (Fig 4.7d and 4.7e), whilst Icam-1 (Fig 4.7d) and Kdr/Vegfr2 (Fig 4.7e) expression were significantly decreased in rBMSC<sup>eNOS-GFP+CAV-1WT</sup> and in rBMSC<sup>eNOS-GFP+CAV-1F92A</sup> cells treated with 2mM L-NAME.
Figure 4.7. Nitric oxide promotes angiogenic responsive gene expression. Relative mRNA expression of the angiogenesis-related genes (a) Vegf-a, (b) Fgf2, (c) Pdgfra and (d) Icam-1, (e), Fgfr2 and (f) Kdr1 were upregulated in eNOS-GFP and eNOS-GFP+CAV-1^{F92A} transduced rBMSCs as assessed by quantitative real time PCR. *p<0.05 and †p<0.05 vs. eNOS-GFP+CAV-1^{F92A} (L-NAME), eNOS-GFP+CAV-1^{WT}, CAV-1^{WT}, CAV-1^{F92A}, GFP and rBMSC. (n=3). One way ANOVA followed by post hoc.
4.4.8. Nitric oxide induces an arterial specific endothelial cell transcriptional profile

To determine the specific type of endothelial cell that is generated through NO signalling, the expression profile of arterial specific transcription factors in which Notch signalling plays a major role was analyzed by RT-qPCR. The mRNA expression pattern of Dll4, Notch1 and Notch4, upstream mediators of Notch signalling, and Hey2, a downstream effector of Notch signalling and arterial EC specific ephrinB2 were assessed by quantitative real time PCR. A significant up regulation of the Dll4, Notch 1 and Hey2 (Fig 4.8) was seen in rBMSC\textsuperscript{eNOS-GFP} and this was further increased in rBMSC\textsuperscript{eNOS-GFP+CAV-1F92A} cells. Treatment with 2mM L-
NAME and overexpression of eNOS-GFP and CAV-1\textsuperscript{WT} in rBMSCs (rBMSC\textsuperscript{eNOS-GFP+CAV-1WT}) resulted in a significant reduction of Dll4, Notch 1 and Hey2 (Fig 4.8) expression. On the other hand, Notch4 and the mature arterial endothelial marker ephrinB2 were not detected in any of the cells (Fig 4.8)
Figure 4.8. Nitric oxide induces arterial specific gene expression. Relative mRNA expression of arterial specific transcription factors Dll4, Notch1, and Hey2 were upregulated in eNOS-GFP and eNOS-GFP+CAV-1<sup>F92A</sup> transduced rBMSCs as assessed by quantitative real time PCR. Notch 4 and Ephrinb2 were not detected. *p<0.05 and †p<0.05 vs. eNOS-GFP+CAV-1<sup>F92A</sup> (L-NAME), eNOS-GFP+CAV-1<sup>WT</sup>, CAV-1<sup>WT</sup>, CAV-1<sup>F92A</sup>, GFP and rBMSC. (n=3). One way ANOVA followed by post hoc
4.4.9. Nitric oxide inhibits venous and lymphatic master transcription factors

In order to determine the dynamics of the venous and lymphatic endothelial cell transcriptional profiles in response to NO signalling, the expression patterns for the venous specific master transcription factor orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor II (Coup-tflII) and a lymphatic specific transcription factor prospero homeobox protein 1 (Prox1) were assessed. Interestingly, both Coup-tflII (Fig 4.9a) and Prox1 (Fig 4.9b) mRNA expression was decreased in rBMSC\textsuperscript{eNOS-GFP} and they were further down regulated in rBMSC\textsuperscript{eNOS-GFP+CAV-1F92A}. In contrast, this reduced expression of both Coup-tflII and Prox1 was not seen in rBMSC\textsuperscript{eNOS-GFP+CAV-1WT} and 2mM L-NAME treated rBMSC\textsuperscript{eNOS-GFP+CAV-1F92A} compared to rBMSC\textsuperscript{eNOS-GFP+CAV-1F92A} suggesting that increased levels of NO may inhibit venous and lymphatic specific master transcription factors expression. On the other hand, none of the mature venous endothelial markers, EphB4, Lefy1, Lefty2 (Fig 4.9a) and lymphatic endothelial marker Lyve1 and transcription factor Sox18 (Fig 4.9b) were detected in any of the cells.
Figure 4.9. Nitric oxide inhibits venous and lymphatic master transcription factor gene expression. Relative mRNA expression of the (a) venous specific master transcription factor Coup-TFI1 and mature markers EphB4, Lefty1 and Lefty2, and (b) lymphatic specific transcription factors Prox1, Sox18 and mature marker Lyve1 expression was assessed by quantitative real time PCR. #p<0.05 vs. eNOS-GFP, eNOS-GFP+CAV-1^{F92A} (L-NAME), eNOS-GFP+CAV-1^{WT}, CAV-1^{WT}, CAV-1^{F92A}, GFP and rBMSC. (n=3). One way ANOVA followed by post hoc.
4.4.10. Nitric oxide promotes endothelial differentiation of rBMSCs through canonical Wnt signalling.

To examine the role of canonical Wnt signalling during NO mediated endothelial differentiation, expression of Wnt3a was assessed by quantitative real-time PCR. Expression of the canonical Wnt ligand Wnt3a (Fig 4.10a) was up regulated in rBMSC<sup>eNOS-GFP</sup> and significantly further increased in rBMSC<sup>eNOS-GFP+CAV-1F92A</sup> (Fig 4.10a). Treatment with 2 mM L-NAME showed down regulation of Wnt3a expression in rBMSC<sup>eNOS-GFP+CAV-1F92A</sup> (Fig.4.10a), indicating that NO induces canonical Wnt3a expression in rBMSCs. Furthermore, western blot analysis revealed increased expression of β-catenin in rBMSC<sup>eNOS-GFP</sup> and interestingly stronger expression was observed in rBMSC<sup>eNOS-GFP+CAV-1F92A</sup> (Fig 4.10c). Treatment of rBMSC<sup>eNOS-GFP+CAV-1F92A</sup> with 2 mM L-NAME resulted in a loss of β-catenin protein expression (Fig 4.10c).

To further analyse the relationship between NO-induced endothelial differentiation and Wnt3a signalling, rBMSC<sup>eNOS-GFP+CAV-1F92A</sup> were treated with 20 ng/ml of the Wnt signalling inhibitor, Dickkopf-related protein 1 (Dkk-1). Dkk-1 treatment resulted in a significant down regulation of the endothelial specific marker Cd31 (Fig 4.10b) compared to untreated rBMSC<sup>eNOS-GFP+CAVF92A</sup>.
Figure 4.10. Nitric oxide promotes endothelial reprogramming through canonical Wnt signalling. Relative mRNA expression of the (a), canonical Wnt3a and (b), Cd31 were upregulated in eNOS-GFP and eNOS-GFP+CAV-1<sup>F92A</sup> transduced rBMSCs as assessed by quantitative real time PCR. (c), Western blot analysis of β-catenin expression. *p<0.05 and #p<0.05 vs. eNOS-GFP+CAV-1<sup>F92A</sup> (L-NAME), eNOS-GFP+CAV-1<sup>F92A</sup> (Dkk-1), eNOS-GFP+CAV-1<sup>WT</sup>, CAV-1<sup>WT</sup>, CAV-1<sup>F92A</sup>, GFP and rBMSC. (n=3). One way ANOVA followed by post hoc.
4.4.11. Nitric oxide inhibits Dnmt1 and histone deacetylase Sirt6.

To understand the mechanism by which nitric oxide may mediate endothelial differentiation of rBMSCs, the expression of chromatin deacetylase Sirt6 and DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b was assessed. RT-qPCR analysis revealed that both Sirt6 (Fig 4.11a) and Dnmt1 (Fig 4.11b) were significantly down regulated in rBMSC<sup>eNOS-GFP+CAV-1F92A</sup>. Interestingly low Sirt6 (Fig 4.11a) and Dnmt1 (Fig 4.11b) expression were returned to basal levels with 2mM L-NAME treatment in rBMSC<sup>eNOS-GFP+CAV-1F92A</sup>, and rBMSC<sup>eNOS-GFP+CAV-1WT</sup> which were similar to un-transduced rBMSC<sup>WT</sup>, CAV-1<sup>WT</sup> transduced rBMSCs (rBMSC<sup>CAV-1WT</sup>), CAV-1<sup>F92A</sup> delivered rBMSCs (rBMSC<sup>CAV-1F92A</sup>), and rBMSC<sup>GFP</sup> suggesting that NO may play an important role in epigenetic modification. Of note, there was not any significant difference in Dnmt3a and Dnmt3b expression observed among the treatment groups (Fig 4.11b).
Figure 4.11. Nitric oxide inhibits chromatin deacetylase and DNA methyltransferase expression. (a), histone deacytlylase Sirt6 and (b) DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b mRNA expression. *p<0.05 vs. eNOS-GFP, eNOS-GFP+CAV-1F92A (L-NAME), eNOS-GFP+CAV-1WT, CAV-1WT, CAV-1F92A, GFP and rBMSC. (n=3). One way ANOVA followed by post hoc
4.4.12. *In vivo* blood vessel formation with NO producing rat BMSCs

The vasculogenic potential of the lentiviral vector transduced rBMSCs was evaluated through sub-cutaneous transplantation of cells in a biomaterial scaffold and new *in vivo* blood vessels formation was evaluated after 1 week implantation. To evaluate blood vessel formation, the genetically engineered cells were suspended in fibrin and seeded into 6 mm polyurethane scaffolds prior to transplantation. Following this, the cells containing scaffolds were transplanted under the skin of the dorsal side of nude rats. After 7 days, the scaffolds were harvested, paraffin embedded and stained (Fig 4.12). Staining with haematoxylene and eosin (H&E) (Fig 4.13a) and blood vessels specific lectin (Fig 4.13b) demonstrated that implanted cells formed blood vessels regardless of the genetic modification and erythrocytes also could be observed in newly formed blood vessels suggesting that those vessels may have been associated with the host circulation system (Fig 4.13). However, when the blood vessels formation was quantified there was no significant difference seen among the groups (Fig 4.13c).

4.4.13. Non-specific staining with CD31 antibody

The fixed and processed implanted cells were also stained with endothelial specific rat Cd31 antibody to observe Cd31 expression. However, the staining with Cd31 was not specific as all cells were positive for Cd31 (Fig 4.14). Subsequently, blood vessel formation was quantified with lectin staining (Fig 4.13c).
Figure 4.12. Procedure for transplantation of genetically modified rBMSCs. Genetically modified and un-modified rBMSCs were resuspended in fibrinogen and thrombin followed by seeded on polyurethane scaffolds. After 1 h incubation on 37°C in 5% CO₂ incubator, the scaffolds were transplanted under the skin of dorsal side of nude rats and after 7 days, the scaffolds were harvested and processed.
Figure 4.13. Immunohistological analysis of transplanted cells. After 7 days of post transplantation, the scaffolds were harvested and paraffin embedded as described in materials and methods. Following, the paraffin embedded sections were stained with (a) Hematoxylin and eosin stain (H&E) and (b) blood vessels specific lectin. Black arrow and white triangles indicate blood vessels and blue arrow and red triangle indicate scaffolds. (c), Quantification of vascular volume. (n=3). Scale bars=0.2 mm
Figure 4.14. Immunohistochemistry analysis of Cd31 expression of transplanted cells. After 7 days post transplantation, the cell-scaffolds were stained with rat specific CD31 antibody. White arrow indicates the vessels like structures and red arrow indicates the scaffold.
4.4.14. Survival of eNOS engineered rBMSCs and formation of blood vessels
in vivo

To investigate whether the genetically engineered cells survived within the scaffolds and formed blood vessels in vivo, the fixed and processed transplanted cells were stained with a GFP specific antibody since only the eNOS-GFP genetically modified cells would express GFP. It was observed that some of the transplanted cells survived during the transplantation period (7 days) as the GFP expression was observed only in transplanted cells (Fig 4.15) and interestingly, some of the blood vessels were shown association with GFP positive cells in eNOS-GFP+CAV-1F92A treatment group (Yellow arrow in Fig 4.15), suggesting that these nitric oxide producing cells may form blood vessels in vivo.
Figure 4.15. eNOS-GFP genetically modified cells form blood vessels in vivo. After 7 days of post transplantation, the scaffolds were harvested and paraffin embedded as described in materials and methods. Following, the paraffin embedded sections were stained with a GFP antibody. White arrow indicates the GFP positive cells and yellow arrow indicate the newly formed blood vessel associated with GFP positive cells.
4.5. Discussion

The major finding from the current study is the demonstration that, engineering of eNOS-NO signalling pathways in rBMSCs can be successfully used to generate endothelial like cells from rBMSCs. This approach has been demonstrated through a series of experiments, whereby lentiviral vector overexpression of eNOS together with a mutant caveolin-1 (CAV-1F92A) enhanced eNOS phosphorylation and NO production resulting in rBMSC conversion to an endothelial like lineage. Finally, transplantation of rBMSC<sup>eNOS-GFP+CAV-1F92A</sup> resulted in the formation of <i>in vivo</i> blood vessels as evident by lineage tracing with GFP staining (Fig 4.15).

NO has been shown to participate in neoangiogenesis, in which Ding et al demonstrated that stimulation of endoplasmic reticulum protein, Calreticulin (CRT) can increase the angiogenic potential of HUVECs through increasing NO production via increased eNOS phosphorylation and inhibition of eNOS attenuated this process (Ding et al. 2014). Similarly, Chen and colleagues revealed that phosphorylation of eNOS at Ser-633 by protein kinase Pim1 increased NO production and angiogenesis (Chen et al. 2016). Interestingly, it has been shown that iNOS and nNOS are expressed in rBMSCs but eNOS is absent (Gomes et al. 2013) and inhibition of iNOS and nNOS has been shown to promote vasculogenesis whereas inhibition of eNOS in HUVEC cells has been shown to attenuate it. This highlights the opposing roles of NOS isoforms in terms of vasculogenesis in which eNOS can promote whilst iNOS and nNOS inhibit the blood vessels generation.
eNOS phosphorylation at Ser-1179 is an important step in the functioning of eNOS-NO signalling (Sessa, 2004). Increased phosphorylation status at Ser-1179 residues in eNOS and CAV-1F92A delivered rBMSCs (Fig 4.4a) may suggests that positive feedback mechanisms of NO in which increased levels of NO synthesis through eNOS-CAV-1F92A interaction may induce Akt expression (Fig 4.4a) as a downstream target of NO (Kawasaki et al. 2003) and increased expression of Akt may promote eNOS phosphorylation as a positive feedback loop (Sessa, 2004). However, the mechanism remains to be explored to understand the increased eNOS phosphorylation when CAV-1F92A is available. CAV-1 has been shown to be an endogenous negative regulator of eNOS and can therefore attenuate eNOS derived NO synthesis (Garcia-Cardena et al. 1997; Bernatchez et al. 2005, Bernatchez et al. 2011). Of note, CAV-1 is naturally expressed in MSCs (Baker et al. 2015) and therefore reduced eNOS activation and subsequent NO production was observed in eNOS delivered cells (Fig 4.4) and on the other hand overexpression of mutated version of CAV-1 (CAV-1F92A) with eNOS was attributed with increased levels of NO synthesis (Fig 4.4) which may be due to the interaction of the F92A mutation with the eNOS reductase domain and this interaction may increase electron mobility by removal of aromatic rings (phenyl alanine contains aromatic rings in its side chain but alanine does not) which may reduce electron acceptance by CAV-1F92A and therefore activate eNOS by electron mobilization through reductase domain (Bernatchez et al. 2005).

Wnt signalling has been shown as a key developmental pathway in vasculogenesis (Corada et al. 2010) and in this regard, canonical Wnt3a has been shown to
promote angiogenesis by promoting VEGF signalling (Gore et al. 2011). In the activated canonical Wnt pathway, Wnt3a associated with cell surface frizzled receptors, which resulted in β-catenin stabilization in the cytoplasm by inhibiting its phosphorylation (Voloshanenko et al. 2013) and promote subsequent translocation into the nucleus (Voloshanenko et al. 2013). In the nucleus, β-catenin associates with TCF/LEF region to initiate the transcription of angiogenic responsive genes such as IL 8 (Masckauchan et al. 2005). On the other hand increased levels of NO has been shown to induce Wnt/β-catenin signalling by inhibiting Dkk-1 (Du et al. 2013), and therefore the observed induction of endothelial differentiation by \( rBMC^{eNOS-GFP+CAV-1F92A} \) cells (Fig 4.5 to 4.7) may link with induction of Wnt/β-catenin signalling (Fig 4.10). Together, it is a possible explanation that nitric oxide may stabilize β-catenin for its nuclear translocation through activation of Wnt3a directed canonical Wnt signalling which may result in increased endothelial specific gene transcription.

Endothelial cells type development including arterial, venous and lymphatic specification is tightly regulated by transcriptional programs (Zhong et al. 2000; Herzog et al., 2001). Notch signalling induces arterial specification (Duarte et al., 2004; Krebs et al. 2004) and genetic deletion of Notch pathway components has been shown to inhibit arterial development (Kokubo et al. 2005; Koo et al. 2005). Coup-tfII (Chicken ovalbumin upstream promoter transcription factor 2), also known as NR2F2 (nuclear receptor subfamily 2, group F, member 2), is the master transcription factor in venous specification and has been shown to suppress Notch signalling to direct endothelial differentiation towards venous identity (You et al. 2004).
2005). On the other hand, it has been demonstrated that sub set of venous endothelial cells start to express lymphatic specific master transcription factor Prox1 directing that cell population towards lymphatic identity (Francois et al. 2008). It was found that eNOS-NO signalling inhibits venous and lymphatics specific master transcription factors Coup-tfII and Prox1, and interestingly increased arterial specifying Notch signalling associated components, in which upstream Notch component Dll4, Notch1 and downstream Notch target Hey2 (Fig 4.8). Interestingly, Corada et al have shown that Wnt/β-catenin signalling acts upstream to the Notch signalling and β-catenin can significantly increase Notch signalling both in vitro and in vivo (Corada et al. 2010). Thus, it is a possible explanation that eNOS-NO signalling may up regulate Wnt/β-catenin signalling to direct the rBMSC commitment towards arterial endothelial identity by inhibiting venous specific EphB4 and lymphatic master regulator Prox1 through inhibiting Coup-tfII since expression of venous specific Coup-tfII is required for venous specification (Aranguren et al. 2013; Chen et al. 2012) and initiation of Prox1 expression by direct interaction (Yamazaki et al. 2009; Srinivasan et al. 2010).

Epigenetic regulation of chromatin can be driven by both DNA and histone modifying proteins (Strahl and, Allis, 2000; Kouzarides, 2007). DNA methyltransferases such as Dnmt1 and Dnmt3a are DNA modifying proteins which can add a methyl group to DNA and this is often associated with inhibition of neighboring gene transcription (Liu et al. 2016). Therefore, DNA methyltransferases are important components in the regulation of cellular differentiation especially their expression in human bone marrow MSCs has been
shown to repress endothelial differentiation (Zhang et al. 2016) and siRNA mediated knockdown of Dnmt1 and Dnmt3a have been shown to promote arterial endothelial differentiation. Interestingly, methylation status of the eNOS promoter has been shown to be involved in EC identity, in which, it has been demonstrated that demethylation of the eNOS promoter in EC and on the other hand the eNOS promoter has been shown to be methylated in non-endothelial smooth muscle cells (Chan et al. 2004; Fish et al. 2005), indicating the importance of DNA methylation for endothelial cell identity, and furthermore inhibition of DNA methylation by inhibiting Dnmt through 5'-aza-2'-deoxycytidine (aza-dC) treatment in ECs has been shown to promote endothelial differentiation (Banerjee and Bacanamwo, 2010). Histones can be modified by posttranslational covalent modifications such as acetylation (Strahl and Allis, 2000; Kouzarides, 2007; Zhang and Reinberg, 2001) and methylation (Hickok et al. 2013) at their N-terminal tails. During histone acetylation, an acetyl group is cleaved from acetyl-coenzyme A, is covalently linked to lysine residues and this process is catalysed by histone acetyltransferases (Carrozza et al. 2003). This modification can reduce the positive charge on lysine residues which subsequently decrease the affinity of histones for negatively charged DNA and results in DNA being more accessible for binding of transcription factors and co-activators resulting in increasing the neighboring gene transcription (Nott and Riccio, 2009). Conversely, histone deacetylation is the process of removal of acetyl groups from histone and is catalysed by histone deacetylases (HDACs) which results in a closed chromatin conformation leading to transcriptional repression (Verdin et al. 2003; Grozinger and, Schreiber, 2002).
Nott and colleagues have demonstrated that elevated levels of nitric oxide in neuron progenitor cells can inhibit HDAC2 enzymatic activity by nitrosylation (Nott et al. 2008), and inhibition of HDAC2 can promote neuron specific Bdnf gene transcription and resulting in increased neuronal differentiation. Furthermore, Sirt6 is another member of the HDAC family and has been shown to participate in ESC differentiation. Etchegaray and colleagues recently demonstrated that deletion of SIRT6 from ESCs could induce differentiation towards a neuronal lineage (Etchegaray et al. 2015). Interestingly, Wang et al reported that expression of Sirt6 in hematopoietic stem cells (HSCs) represses Wnt signalling associated genes and deletion of Sirt6 in HSCs could activate Wnt signalling gene transcription (Wang et al. 2016). Together, the findings from this current study may suggest that inhibition of Sirt6 (Fig 4.11a) and Dnmt1 (Fig 4.11b) by increased levels of NO may attribute to increased endothelial differentiation.

Finally, in vivo transplantation of NO producing cells (rBMSC^eNOS-GFP and rBMSC^eNOS-GFP+CAV-1F92A) did not display enhanced blood vessel formation (Fig 4.13) which was not the phenomenon seen in vitro where enhanced endothelial differentiation was observed. The possible reasons for this difference between in vitro and in vivo settings might be due to the extracellular matrix (ECM) fibrin used for in vivo transplantation. Lozito et al have shown that use of matrigel with MSCs can stimulate their differentiation towards an endothelial phenotype (Lozito et al., 2009), in which they have demonstrated that the crosslinking level of the matrix significantly induced endothelial differentiation of MSCs. In addition, Osawa et al have demonstrated that endothelial specific Cd31 expression can be a
mechanoresponsive molecule in which the expression of Cd31 has been shown to be responsive for the elasticity of the ECM (Osawa et al. 2002). Furthermore, Chung and colleagues have shown significant increase in angiogenic responsive gene expression, von Willebrand factor and VEGF in adipose derived MSCs when cultured on fibrin compared to normal MSCs (Chung et al. 2015). Several other studies have also demonstrated that fibrin acts as a stimulator for angiogenic factors such as Vegf release (Morin and Tranquillo, 2013; Shiose et al. 2004). Another possible explanation might be the increased cell to cell contact when seeded onto fibrin which may also promote endothelial differentiation in vivo regardless of their in vitro modification (Janeczek Portalska et al. 2012). However, it is noteworthy that, the transplantation period of this study was relatively short (7 days) and therefore, this limited transplantation period might not be enough to see the significant effects of NO producing cells on blood vessel formation.

Together, these findings may support a paradigm that eNOS-NO signalling may modulate epigenetic regulation of chromatin by inhibiting both DNA methylatransferase Dnmt1 and histone deacetylase Sirt6 transcriptional repressors, which may induce transcriptionally active chromatin conformation resulting in activated Wnt/β-catenin signalling which may promote downstream arterial endothelial specific genes transcription (Fig 4.16). However, further validation is needed in terms of the role of Dnmt1 and Sirt6 in endothelial differentiation of BMSCs by genetic deletion of both Dnmt1 and Sirt6 to show increased differentiation. In addition, further experiment such as chromatin
immunoprecipitation, ChIP-seq would be better platform to draw comprehensive conclusion to demonstrate NO mediated epigenetic modifications.

4.6. Conclusions

In summary, findings from this study provide an insight into the role of NO in lineage conversion of MSCs to endothelial like cells through activation of Wnt/β-catenin signalling pathway. This may lead to the development of novel cell-based therapeutic approaches for vascular repair, in particular in vitro modification of MSCs to produce NO and for subsequent transplantation into damaged areas to improve vascularization and wound healing.

![Diagram](image)

**Figure 4.16. Proposed epigenetic mechanisms underlying the endothelial differentiation governed by NO in rBMSCs.** Molecular control of NO levels may inhibit endogenous histone deacetylase *Sirt6* and DNA methyltransferase *Dnmt1* to convert transcriptional inactive conformation of chromatin state to transcriptional active chromatin state which may activate arterial specific transcriptional network.
4.7. References


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Chapter 5: Reconstitution of the endothelial nitric oxide synthase signalling pathway in fibroblasts and adipose derived stem cells can enhance transcription factor mediated cardiac reprogramming.
5.1. Abstract

Direct cellular reprogramming of fibroblasts into cardiomyocytes like cells (CLCs) by forced expression of the lineage specific transcription factors, Gata4, Mef2C, and Tbx5 (termed as GMT) has been previously demonstrated. However, this approach is inefficient in human cells in particular fibroblasts. This chapter describes the genetic modification of human foreskin fibroblasts (BJ) and equine adipose derived stem cells to express eNOS and enhanced NO signalling to promote GMT mediated cardiac reprogramming. Nitric oxide (NO) is an important signalling molecule which has been shown to promote differentiation of embryonic stem cells to cardiomyocytes and endothelial nitric oxide synthase (eNOS) has been shown to promote embryonic cardiac development. To enhance NO production, the eNOS-CAV-1WT interaction was modified to be non-inhibitory by co-expressing a mutated caveolin-1 protein (CAV-1F92A) together with eNOS. Co-expression of eNOS and CAV-1F92A increased the production of NO significantly compared to eNOS alone, and treatment with eNOS inhibitor L-NAME or co-expression of eNOS with CAV-1WT significantly abrogated NO synthesis.

The effect of NO on cardiac reprogramming in GMT expressing cells was monitored by GFP expression through a cardiac specific αMHC promoter also delivered by lentiviral vector in transduced cells. The data shows that αMHC-GFP expression appeared after 2 days with further increasing numbers of fluorescent cells for up to 2 weeks showing a cardiomyocyte like morphology combined with α-actinin immunostaining in cells co-expressing eNOS and CAV-1F92A and GMT driven reprogramming compared to cells without NO production. Specifically a
binucleated morphology was observed in BJ fibroblasts transduced with GMT+eNOS+CAV-1F92A suggesting that NO signalling may promote reprogramming of distinct type of cardiomyocytes. The NO driven reprogramming of fibroblasts was further investigated by RT-qPCR and flowcytometry analysis showed increased expression of cardiac specific transcripts and cardiac troponin T (cTnT) protein respectively. Conversely, inhibition of eNOS-NO signalling with L-NAME or with CAV-1WT attenuated CLC generation. NO may enhance cardiac reprogramming through canonical Wnt signalling and these findings may provide new strategies to enhance transcription factor based generation of cardiomyocytes for clinical applications.
5.2. Introduction

Coronary heart diseases are a main cause of death worldwide and are associated with loss or dysfunction of cardiomyocytes (Lopez et al. 2006). The mammalian heart is composed of several types of cells including resident cardiac stem cells, smooth muscle cells, endothelial cells, fibroblasts and cardiomyocytes (CMs) (Porter and Turner, 2009). CMs have a limited capacity to regenerate (Laflamme and Murry, 2005; Mercola et al. 2011) and the only long-term therapeutic option is heart transplantation which is limited due to a lack of organ donation.

In 2006, Yamanaka’s laboratory demonstrated that terminally differentiated fibroblasts can be reprogrammed to a pluripotent state by forced expression of four transcription factors named Oct3/4, cMyc, Sox2 and Klf4 (Takahashi and Yamanaka, 2006). In subsequent studies several groups have demonstrated that fibroblasts can also be reprogrammed directly into various lineages such as neurons and hepatocytes by forced expression of specific transcription factors (Vierbuchen et al. 2010, Huang et al. 2011). In a landmark study, Ieda et al (2010) demonstrated that a combination of three cardiac transcription factors, Gata4, Mef2c, and Tbx5 (termed as GMT) could reprogram dermal or cardiac fibroblasts to cardiomyocyte-like cells (CLCs). Lineage tracing approaches have revealed that GMT mediated fibroblast reprogramming do not pass through a mesodermal or cardiac progenitor stage (Ieda et al. 2010). Since GMT transcription factor based in vitro reprogramming is inefficient, other combinations of factors have been tested, such as Hand2 (Song et al. 2012) to improve efficiency and also the use of miRNAs such as the muscle-specific miRNAs, miR-1 and miR-133.
(Muraoka et al. 2014). In addition, employing a four micro RNA combination, miR-1, miR-133, miR-208, and miR-499 has been shown to convert mouse fibroblasts to CLCs without the use of any exogenous transcription factors (Jayawardena et al., 2012) and this group further showed the efficiency of micro RNA based reprogramming was further increased when combined with the JAK1 inhibitor (Jayawardena et al. 2012). Furthermore, modification of Mef2C activity by adding the MyoD transactivation domain can significantly enhance trans-differentiation efficiency of fibroblasts when combined together with Gata4, Hand2, Mef2C and Tbx5 factors (GHMT) (Hirai et al. 2013).

Modulation of cellular signalling pathways can also promote reprogramming, and inhibition of TGF-β signalling by the small molecule SB431542 has been shown to promote GHMT driven cardiac reprogramming (Ifkovits et al. 2014, Zhao et al. 2015) whilst activation of canonical Wnt signalling by inhibiting Gsk3 and including the Wnt activator CHIR99021 has been shown to promote fibroblast conversion efficiency when combined with cardiogenic medium which was included with small molecules, activin A and BMP4, and interestingly, in this study only the small molecules were used to reprogram fibroblasts to cardiomyocytes (Cao et al. 2016). Zhao et al (2015) have demonstrated that inhibition of pro-fibrotic signalling with the ROCK inhibitor Y-27632 could significantly increase cardiomyocyte generation. Moreover, activating FGF and VEGF signalling with GMT can markedly increase the yield of beating cardiomyocytes (Yamakawa et al. 2015), and interestingly overexpression of Akt1 (RAC-alpha serine/threonine-protein kinase) has been
shown to promote cardiac reprogramming of mouse embryonic fibroblasts (Zhou et al. 2015). Finally, Hirai et al demonstrated that modifying chromatin by adding GSK126, an inhibitor for EZH2 methyltransferase and UNC0638 an inhibitor for G9a histone methyltransferase could further enhance GHMT mediated cardiac reprogramming (Hirai et al. 2013). More recently, Bmi1 has been revealed as a key epigenetic barrier for direct cardiac reprogramming and inhibition of Bmi1 increases fibroblast to cardiomyocyte conversion efficiency (Zhou et al. 2016).

However, the efficiency of direct reprogramming of human fibroblasts, into beating cardiomyocytes is still less than 0.1% as previously reported (Muraoka et al. 2014; Wang et al. 2015; Hirai et al. 2013; Hirai et al. 2014, Ifkovits et al. 2014), suggesting that alternative strategies are needed to improve transcription factor based cardiomyocyte generation.

Nitric oxide (NO) is a diatomic free radical gas which can mediate a variety of physiological events such as smooth muscle relaxation, vasodilatation, neurotransmission, inhibition of platelet aggregation, immunomodulation (Beckman et al. 1996; Bryan et al. 2009), and cardiac differentiation of ESCs (Mojoo et al. 2008). Interestingly, Liu et al demonstrated that NOS3/eNOS can promote embryonic development of atrioventricular (AV) valves (Liu et al. 2013). They further showed that induction of AV development through NOS3 is driven by cGMP signalling. NO can regulate cellular differentiation through cyclic GMP (cGMP) dependent mechanisms, epigenetic modification (Hickok et al. 2013; Spallotta et al. 2010) and modulating other cellular signalling pathways such as Notch (Charles et al. 2010), Wnt (Du et al. 2013). Mujoo et al (2008) have demonstrated that NO
signalling components are significantly up regulated during the cardiomyogenesis in mouse embryos, and increased expression of eNOS is evident during ES cell differentiation. The importance of NOS activity and NO during cardiomyogenesis has been demonstrated using murine ES-cell-derived cardiomyocytes, where treatment of the ES-cell-derived cardiomyocytes with the NOS inhibitor L-NAME resulted in decreased development of distinct cross-striation patterns in the cells while addition of an NO donor reversed this effect (Bloch et al. 1999) suggesting the possibility of a direct role of NO for cardiomyocyte maturation. Furthermore, treatment of ES cells with an exogenous NO donor has been shown to promote cardiomyogenesis (Kanno et al. 2004). Expression of iNOS and eNOS begins at E8.5, and interestingly, iNOS expression has been shown to diminish at the later time points while eNOS expression is not reduced, providing further evidence of NO signalling during the cardiomyogenesis (Bloch et al. 1999). Impaired myocardial angiogenesis has been observed in eNOS deficient mice (Zhao et al. 2002), and eNOS knockout neonates display developmental defects in aortic valves (Lee et al. 2000) and atrial and ventricular septal defects (Feng et al. 2002). These studies strongly suggest an important role for nitric oxide in cardiac development pathways and support a potential role for NO signalling in direct cardiac reprogramming of fibroblasts or adult stem cells. In this chapter experiments are described where eNOS-NO signalling through an eNOS-CAV-1<sup>F92A</sup> interaction can promote GMT mediated human dermal fibroblast and adult stem cell conversion into cardiomyocyte like cells by promoting canonical Wnt signalling and these findings may improve cardiac reprogramming strategies.
5.3. Materials and methods

5.3.1. Cell culture

BJ human neonatal foreskin fibroblast cells (ATCC, Manassas, VA), Human embryonic kidney 293T cells (HEK293T) (ATCC, Manassas, VA) and equine adipose derived mesenchymal stem cells (eASCs) (Chapter 3) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (Lonza), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Growth medium) at 37ºC and maintained at 5 % CO₂.

5.3.2. Lentiviral vectors

The list of lentiviral vectors used in this study was listed in table 5.1.

Table 5.1. Lentiviral vectors and reporter constructs used in this study

<table>
<thead>
<tr>
<th>Lentiviral vector</th>
<th>Relevant properties</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWPT-eNOS</td>
<td>CMV-eNOS</td>
<td>This study</td>
</tr>
<tr>
<td>pWPT-CAV-1&lt;sup&gt;F92A&lt;/sup&gt;</td>
<td>CMV-CAV-1&lt;sup&gt;F92A&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pLVX-CAV-1&lt;sup&gt;W1&lt;/sup&gt;</td>
<td>CMV-CAV-1&lt;sup&gt;W1&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>FUW-TetO-Gata4</td>
<td>TetO-Gata4</td>
<td>Addgene</td>
</tr>
<tr>
<td>pLenti6.2/V5-Mef2C</td>
<td>CMV-Mef2C</td>
<td>DNAsu repository</td>
</tr>
<tr>
<td>pLenti6.2/V5-Tbx5</td>
<td>CMV-Tbx5</td>
<td>DNAsu repository</td>
</tr>
<tr>
<td>FUW-M2rtTa</td>
<td>CMV-rtTA</td>
<td>Addgene</td>
</tr>
<tr>
<td>αMHC-eGFP-Rex-Neo</td>
<td>αMHC-eGFP</td>
<td>Addgene</td>
</tr>
</tbody>
</table>
5.3.3. Lentiviral vector production and transduction

Lentiviral vectors used in this study (Table 5.1) were generated by four plasmid transfection of HEK293T cells as described in the methods chapter of this thesis (Section 2.4, Chapter 2) and used transgene expressing lentiviral vectors to transduce BJ cells and eASCs.

5.3.4. Cardiac reprogramming

To investigate the effect of NO signalling on cardiac reprogramming, BJ or eASCs were first seeded in duplicate onto 0.1% gelatin coated wells in 12-well polystyrene tissue culture plates at a concentration of 30,000 cells / well followed by transduction with an αMHC-GFP reporter lentiviral vector and a lentiviral vector expressing the tetracycline reverse trans activator (rtTA) and incubated at 37°C for 48 h. These cells were then further transduced with combinations of lentiviral vectors expressing, GATA4, MEF2C, TBX5, eNOS, CAV-1<sup>F92A</sup>, CAV-1<sup>WT</sup>, and CAV-1<sup>F92A</sup> and incubated for another 72 h in growth medium. The transduced cells were switched to differentiation medium (Growth medium + 25 µg/ml L-ascorbic acid + 2 µg/ml doxycycline) and incubated for 14 days with media changed at every 2-3 days (Fig 5.2).
5.3.5. Flowcytometry analysis

For flowcytometry based analysis of cTnT expression, BJ cells were transduced with lentiviral vectors described above (except for the αMHC-GFP reporter) followed by incubation in differentiation medium for up to 14 days. The cells were then harvested, and washed with FACS buffer (PBS containing 5% FBS and 10 μM of rock inhibitor, Y-27632) and fixed in 4% paraformaldehyde in PBS for 10 min with vortexing intermittently in order to maintain a single cell suspension followed by a further two washes with FACS buffer. The cells were re-suspended in 100 µl of flowcytometry permeabilization/wash buffer 1 (BD Bioscience) together with 5 µl of PE conjugated cTnT antibody (BD Bioscience) followed by incubation at room temperature for 30 min in the dark. The stained cells were then washed two times with flowcytometry permeabilization/wash buffer 1 and this was followed by re-suspension of the cells in 200 µl of FACS buffer and were subjected to FACS analysis using a FACSaria cell sorter (BD Biosciences) with gates were set based on fluorescence minus control sample which lacked primary antibody (cTnT).

5.3.6. Immunocytochemistry and fluorescence microscopy

Immunocytochemical detection of α-actinin expression was performed as follows. Briefly, cells were fixed in 4% paraformaldehyde for 20 min at room temperature, treated with 0.1% Triton-X100 in PBS for 10 minutes, blocked in Ultra V block (Thermo Fisher Scientific) for 10 min at room temperature. This was followed by an overnight incubation with a primary mouse monoclonal, anti α-actinin antibody
(1:500, Abcam), and subsequently with an anti-mouse IgG secondary antibody conjugated with Alexa 488 (green) (for cells transduced without αMHC) or Alexa 555 (red) (1:200, Abcam) and Dapi (Nuclear stain) for 1 hour followed by fluorescence microscopy analysis.

5.3.7. Quantitative real time PCR (RT-qPCR)

Total RNA from transduced and control cells after 14 days in differentiation medium was isolated using the TriReagent (Thermo Fisher Scientific) according to the manufacturer’s instructions and the concentration of isolated RNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific), treated with RQ1 RNase free DNase (1 U/1 μg RNA; Promega, WI, USA). cDNA was synthesized with 1 μg RNA from all samples using a High Capacity Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative real-time PCR assays were performed on a QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific) using the TaqMan Universal master mix (Applied Biosystems) with TaqMan probes labeled with a FAM detection probe (Applied Biosystems) ACTC (Hs01109515), TNN2 (Hs00165960), MYH6 (Hs001101145), MYH7 (Hs00165276), MYL2 (Hs00166405), MYL7 (Hs01085598) and endogenous control GAPDH (Hs03929097) (Thermo Fisher Scientific). Assays were performed in duplicate and target gene expression was normalized to human GAPDH mRNA levels using the ΔΔCt method.
For the detection of WNT3A mRNA expression, SYBR detection method was employed using SsoFast EvaGreen Supermix (Bio-Rad) and WNT3A specific primers (Forward primer: TTTGCAGTGACACGCTCAT and reverse primer: CAAACTCGATGTCTCCTGCTA). RT-qPCR assays were performed in duplicate and target gene expression was normalized to human GAPDH mRNA levels detected by human GAPDH specific primers (Forward primer: GTGGACCTGACCTGCGCTTCT, and reverse primer: GGAGGAGTGGGTGTGCCTGT) using the ΔΔCt method.

5.3.8. Reporter assays

For GFP based reporter assays for αMHC-eGFP, the cells were transduced with a lentiviral αMHC-eGFP reporter and were subjected to fluorescence microscopic analysis.

5.3.9. Statistics

Data are presented as mean ± SEM. The statistical significance of differences between two groups was determined by Student’s t test. Statistical analysis for nitric oxide levels, and RT-qPCR data was performed using one way analysis of variance (ANOVA) followed by post hoc. All tests were performed using the statistical software GraphPad Prism 6 (GraphPad, LaJolla, CA, USA). p<0.05 was considered statistically significant.
5.4. Results

5.4.1. The effect of up regulating the eNOS-NO signalling pathway on GMT mediated cardiac reprogramming

Previously it has been demonstrated that GMT mediated human fibroblast reprogramming towards cardiomyocytes is extremely low compared to results in mouse fibroblasts. To assess whether nitric oxide production in target cells could improve efficiency, the eNOS-NO signalling pathway was incorporated in human BJ fibroblasts together with GMT and the conversion efficiency measured by RT-qPCR. Screening of transduced cells showed that transduction of eNOS together with GMT (BJ^{GMT+eNOS}) could significantly induce cardiac specific gene expression, TNN2 (Fig 5.1a) and ACTC (Fig 5.1b) and interestingly co-expression of eNOS with CAV-1^{F92A} (BJ^{GMT+eNOS+CAV-1F92A}) could further increase the GMT mediated cardiac reprogramming (Fig 5.1a and Fig 5.1b) compared to GMT alone (BJ^{GMT}). On the other hand, co-expression of eNOS with CAV-1^{WT} together with GMT (BJ^{GMT+eNOS+CAV-1WT}) did not show any significant difference in TNN2 (Fig 5.1a) and ACTC (Fig 5.1b) expression compared to GMT alone. To further examine whether this enhanced fibroblast conversion efficiency was eNOS mediated, BJ^{GMT+eNOS+CAV-1F92A} cells were treated with the eNOS inhibitor L-NAME (2mM). As expected TNN2 (Fig 5.1a) and ACTC (Fig 5.1b) expression was significantly reduced to levels achieved with GMT alone. When it was tested whether CAV-1^{F92A} or CAV-1^{WT} can have an effect in the absence of eNOS on GMT mediated reprogramming by overexpressing of CAV-1^{F92A} with GMT (BJ^{GMT+CAV-1F92A}) or CAV-1^{WT} with GMT (BJ^{GMT+CAV-1WT}), both TNN2 (Fig 5.1a) and ACTC (Fig 5.1b)
gene expression data showed there was no additive or inhibitory effect on GMT mediated reprogramming. Measurement of nitrite levels in gene modified cells showed that BJ\textsuperscript{GMT+eNOS} and BJ\textsuperscript{GMT+eNOS+CAV-1F92A} significantly increased nitrite levels (Fig 5.1c) and treatment of BJ\textsuperscript{GMT+eNOS+CAV-1F92A} with 2mM L-NAME significantly abrogated NO production in BJ\textsuperscript{GMT+eNOS+CAV-1F92A} cells. In comparison to the other cell types, BJ\textsuperscript{GMT}, BJ\textsuperscript{GMT+eNOS+CAV-1WT}, BJ\textsuperscript{GMT+CAV-1F92A}, and BJ\textsuperscript{GMT+CAV-1WT} or control cells no significant differences in terms of NO synthesis were seen. Since eNOS+CAV-1\textsuperscript{WT}, CAV-1\textsuperscript{WT}, and CAV-1\textsuperscript{F92A} transduced cells did not show any additive or suppressive effect on GMT driven BJ reprogramming, these combinations were excluded from further investigations and used GMT, GMT+eNOS, and GMT+eNOS+CAV-1\textsuperscript{F92A} transduced cells.
Figure 5.1. Relative mRNA expression of cardiac specific genes. (a) *TNN2*, (b) *ACTC* were upregulated in eNOS and eNOS+CAV-1<sup>F92A</sup> transduced BJ and co-expression of eNOS with CAV-1<sup>WT</sup>, and CAV-1<sup>WT</sup> or CAV-1<sup>F92A</sup> alone did not show any additive or suppressive effect on GMT mediated cardiac differentiation as assessed by RT-qPCR. (c), Nitrite levels in transduced cells and controls as measured by Griess assay. *p<0.05 and #p<0.05 vs. GMT+eNOS+CAV-1<sup>F92A</sup> (L-NAME), GMT+eNOS+CAV-1<sup>WT</sup>, GMT, GMT+CAV-1<sup>WT</sup>, GMT+CAV-1<sup>F92A</sup>, induced (BJ in differentiation medium) and non-induced (BJ in growth medium). N=3. One way ANOVA followed by post hoc.
5.4.2. Increased eNOS-NO signalling promotes human fibroblast conversion to αMHC positive cells

When fibroblast conversion to cardiomyocyte-like cells was monitored using a cardiac specific αMHC-eGFP reporter system (Kita-Matsuo et al., 2009), after 2 days in cardiac differentiation medium, GFP+ cells were observed only in BJ\textsuperscript{GMT+eNOS+CAV-1F92A} (Fig 5.3a) suggesting that activated eNOS-NO signalling can promote GMT mediated fibroblast conversion to cardiomyocyte like cells (CLCs). After 14 days in the cardiac differentiation medium, the GFP+ cells were counted and the reprogramming efficiency was found to be 0.13% (Fig 5.3b).
Figure 5.2. Schematic of lentiviral transduction and cardiac reprogramming strategy. Human BJ cells were transduced with αMHC-GFP and rtTA lentiviral vectors and 48 h post transduction, the cells were re-transduced with Gata4, Mef2C, Tbx5, eNOS, CAV-1\textsuperscript{WT} and CAV-1\textsuperscript{F92A} and incubated for 72 h in growth medium followed by switching to cardiac differentiation medium (with or without 2 mM L-NAME). After 14 days of incubation in differentiation medium the reprogrammed cells were subjected to downstream analysis.
Figure 5.3. Activity of a αMHC-eGFP reporter during the cardiac reprogramming. (a), αMHC-eGFP positive cells were detected only in BJ\textsubscript{GMT+eNOS+CAV-1F92A} (b). Quantification of GFP+ cells after 14 days (% of GFP+ cells). *p<0.05 vs. GMT+eNOS, GMT, induced (BJ in differentiation medium) and non-induced (BJ in growth medium). N=3. One way ANOVA followed by post hoc
5.4.3. NO signalling promotes cardiac specific marker gene expression

Expression of cardiac specific markers by RT-qPCR and α-actinin antibody based immunostaining was used to evaluate cardiac reprogramming efficiency. Transduction of eNOS significantly induced cardiac specific ACTC (Fig 5.4b) and TNN2 (Fig 5.4c) gene expression compared to GMT alone and interestingly co-transduction of eNOS with CAV-1$^{F92A}$ further increased them (Fig 5.4b and 5.4c), indicating that that eNOS-NO signalling can induce GMT driven fibroblasts conversion. Furthermore, α-actinin expression was found in BJ$^{GMT}$, BJ$^{GMT+eNOS}$ and BJ$^{GMT+eNOS+CAV-1F92A}$ (Fig 5.4a) cells. In addition, cardimyocytes-like cells contained more than one nucleus (binucleated) reprogrammed by GMT+eNOS+CAV-1$^{F92A}$ gene transduction (Fig 5.5), and this morphology was not observed with other treatments.
Figure 5.4. Nitric oxide enhances cardiac reprogramming of BJ by GMT. (a) Immunocytochemical detection of α-actinin with a cardiac specific α-actinin antibody. Cardiac specific gene expression analysis by RT-qPCR revealed that (b) ACTC and (c) TNN2 were significantly induced in BJ\textsuperscript{GMT+eNOS} and BJ\textsuperscript{GMT+eNOS+CAV-1F92A}. *\(p<0.05\) and \#\(p<0.05\) vs. GMT, induced (BJ in differentiation medium) and non-induced (BJ in growth medium). N=3. One way ANOVA followed by post hoc
Figure 5.5. Binucleated morphology of reprogrammed cells. Immunocytochemistry with a cardiac specific α-actinin antibody showed binucleated positive cells only in BJ reprogrammed by GMT+eNOS+CAV-1F92A.
5.4.4. NO signalling promotes GMT driven cardiac reprogramming efficiency

To estimate the fibroblast conversion efficiency, flowcytometry analysis was employed using a PE conjugated cTnT antibody. cTnT positive cells were only detected in BJ\textsuperscript{GMT+eNOS+CAV-1F92A} with a 0.1% conversion efficiency (Fig 5.6). To confirm whether this increased cardiac reprogramming was eNOS mediated, BJ\textsuperscript{GMT+eNOS+CAV-1F92A} cells were treated with 2 mM \textit{L}-NAME which resulted in a reduction in cTnT+ cells.

5.4.5. eNOS-NO signalling promotes atrial specific gene expression and suppresses ventricular specific gene expression.

Atrial and ventricular specific gene expression patterns during the cardiac reprogramming were measured by RT-qPCR. It was found that atrial specific \textit{MYH6} and \textit{MYL7} (Fig 5.7) were significantly up regulated in BJ\textsuperscript{GMT+eNOS+CAV-1F92A} cells compared to BJ\textsuperscript{GMT+eNOS} and BJ\textsuperscript{GMT}. On the other hand \textit{MYL7} expression was significantly increased in BJ\textsuperscript{eNOS} cells compared to BJ\textsuperscript{GMT} (Fig 5.7). However, \textit{MYH6} (\textit{αMHC}) expression was not seen in BJ\textsuperscript{GMT} or BJ\textsuperscript{GMT+eNOS} cells which strongly matched the \textit{αMHC-GFP} promoter results in BJ\textsuperscript{GMT+eNOS+CAV-1F92A} cells (Fig 5.7). Interestingly, \textit{MYL2} and \textit{MYH7} ventricular marker gene expression was not detected in any of these cells.
Figure 5.6. cTnT expression in reprogrammed cells. Flowcytometry analysis of cTnT+ cells on day 14 in cardiac differentiation medium. Numbers embedded in each graph indicate the percentages of cTnT+ cells revealing that only BJ GMT+eNOS+CAV-1F52A cells expressed cTnT. Induced (BJ in differentiation medium) and non-induced (BJ in growth medium), (SSC; side scatter). N=3.
Figure 5.7. Nitric oxide promotes atrial specific cardiac gene expression. Relative mRNA expression of the atrial specific genes MYH6 and MYL7 were up regulated in GMT+eNOS+CAV-1^F92A transduced BJ cells. Ventricular specific MYL2 and MYH7 gene expression was not detected in any cells. *p<0.05 and #p<0.05 vs. GMT, induced (BJ in differentiation medium) and non-induced (BJ in growth medium). N=3. One way ANOVA followed by post hoc
5.4.6. Nitric oxide may promote cardiac reprogramming through canonical Wnt signalling.

To examine the potential cellular mechanisms involved in GMT driven cardiac reprogramming when eNOS-NO signalling was incorporated, the expression of canonical \textit{WNT3A} was analysed and was up regulated in BJ\textsuperscript{GMT+eNOS} and significantly further increased in BJ\textsuperscript{GMT+eNOS+CAV-1F92A} (Fig 5.8a).

\textit{WNT3A} signalling was also investigated in BJ\textsuperscript{GMTeNOS+CAVF92A} cells which were treated with 20 ng/ml of the Wnt signalling inhibitor, Dickkopf-related protein 1 (Dkk-1) and this resulted in a significant down regulation of the \textit{ACTC} (Fig 5.8b) and \textit{TNN2} (Fig 5.8c) compared to untreated BJ\textsuperscript{GMTeNOS+CAVF92A}.
Figure 5.8. Nitric oxide promotes WNT3A gene expression. Relative mRNA expression of the (a), canonical WNT3A, (b), ACTC and (c), TNN2 were up regulated in BJ$^{\text{GMT+eNOS}}$ and BJ$^{\text{GMT+eNOS+CAV-1F92A}}$ cells as assessed by RT-qPCR. \#p<0.05 vs. GMT+eNOS, GMT, and GMT+eNOS+CAV-1F92A (Dkk-1), induced (BJ in differentiation medium) and non-induced (BJ in growth medium). N=3. One way ANOVA followed by post hoc
5.4.7. eNOS-NO signalling promotes equine adipose derived stem cell reprogramming towards cardiomyocyte-like cells

To investigate whether NO can promote GMT driven cardiac reprogramming in adult stem cells, equine adipose derived mesenchymal stem cells (eASC) were used. In this experiment, the lentivirial transduced cells were re-plated on fibrin gel and the cardiac differentiation medium was supplemented with additional 5 ng/ml BMP2, and found that similar to human fibroblasts, cardiac reprogramming of eASCs was higher in GMT and eNOS transduced cells and this was further enhanced in eASCs transduced with GMT+eNOS+CAV-1$^{F92A}$ as observed with the αMHC-GFP reporter assay (Fig 5.9a). GMT driven cardiac reprogramming of eASC was more efficient than BJ fibroblasts as a higher number of αMHC-GFP positive cells, 0.3% (Fig 5.9b) compared to BJ (0.1%, Fig 5.3b) was seen. Confirming, the generation of cardiomyocyte-like cell, immunostaining analysis showed that, α- actinin expression in GMT, GMT+eNOS, and GMT+eNOS+CAV-1$^{F92A}$ transduced cells (Fig 5.9c) and interestingly sarcomere like structures (Basic unit of the striated heart muscles) which were pointed by white arrows (Fig 5.9c) were seen in GMT+eNOS, and GMT+eNOS+CAV-1$^{F92A}$ transduced cells. When nitrite levels were measured in these cells, a significant increase of nitrite levels were observed in eASC transduced with GMT+eNOS and GMT+eNOS+CAV-1$^{F92A}$ and these increased nitrite levels were maintained during the period of reprogramming (14 days) (Fig 5.9d)
Figure 5.9. Nitric oxide promotes reprogramming of equine adipose stem cells towards cardiomyocyte-like cells. (a) Activity of the lentiviral αMHC-eGFP reporter during the cardiac reprogramming. (b), Quantification of GFP+ cells after 14 days and the % of GFP+ cells was expressed to the initially seeded 30,000 cells. (c), Immunocytochemical detection of α-actinin with a cardiac specific α-actinin antibody. (d), Nitrite measurements. *p<0.05 and #p<0.05 vs. GMT, induced (eASCs in differentiation medium) and non-induced (eASCs in growth medium). N=3. One way ANOVA followed by post hoc.
5.5. Discussion

Direct cellular reprogramming of fibroblasts into cardiomyocytes provides a promising approach for cardiac repair to treat chronic heart disease and acute ischemic heart disease (Liu et al. 2012; Hansson, and Chien, 2012). However, current transcription factor based reprogramming strategies appear to be inefficient especially with human fibroblasts (Muraoka et al. 2014; Wang et al. 2015; Hirai et al. 2013; Hirai et al. 2014, Ifkovits et al. 2014). Nitric oxide plays an important role in cardiac development and hence may act as an enhancer of the reprogramming process, and this chapter demonstrates that reconstitution of the eNOS-NO signalling pathway in human fibroblasts and equine adipose derived stem cells can significantly improve GMT mediated cardiomyocytes like cell formation and this process may be regulated by canonical Wnt signalling.

Despite successful cardiomyocyte reprogramming of mouse fibroblasts using retroviral vector delivery of cardiac specific transcription factors GMT (Ieda et al. 2010), the Olsen group has shown the difficulty of human fibroblast reprogramming to cardiomyocytes, in which they have reported that GMT or GMT and Hand2 (GHMT) is insufficient to generate cardiomyocytes (Nam et al. 2013). They further reported that overexpression of microRNAs (miR-1 and miR-133) together with GHMT, and myocardin (MyoCD) could reprogram human fibroblasts into CLCs highlighting that microRNAs like epigenetic regulators could be useful candidates to improve direct human cardiac reprogramming. NO has been shown to promote histone methylation (Hickok et al. 2013) and histone acetylation (Spallotta et al. 2010) which are the processes associated with transcriptional active open
It could be proposed that NO may induce open chromatin states which may facilitate transcription factors (GMT) to interact more efficiently with cardiac specific gene promoters and subsequently enhance respective gene transcription. It is also possible that this effect may further be enhanced by increased NO bioavailability in the reprogramming environment through over expression of eNOS and CAV-1$^{F92A}$ where F92A mutation has been shown to increase eNOS derived NO production (Bernatchez et al. 2005). Supporting this hypothesis, it has been shown that cardiac differentiation of human MSCs is enhanced with 5-Azacytidine (5-aza) treatment (Qian et al. 2012b) and importantly, 5-aza has been shown to promote histone acetylation through up regulation of histone acetyltransferase 1 (HAT1) and down-regulation of histone deacetylase enzyme 1 (HDAC1) in human skin fibroblasts (Manzoni et al. 2016).

In this study NO signalling significantly induced cardiac reprogramming and interestingly appeared to favorably reprogram towards atrial like cardiomyocytes as observed by qPCR analysis (Fig 5.7). A recent study has reported that addition of an Akt 1 transgene to the GHMT cocktail increased atrial gene expression such as Myh6 during cardiac reprogramming of mouse fibroblasts (Zhou et al. 2015). Furthermore, binuclear cardiomyocytes were generated when BJ cells were reprogrammed with GMT and eNOS+CAV-1$^{F92A}$ (Fig 5.5). Zhou et al (2015) also reported that addition of Akt 1 transgene into the GHMT mixture resulted in binucleated or multi nucleated cells which was rarely observed in GHMT alone treated cells (Zhou et al. 2015). Protein kinase B or Akt is the enzyme responsible for phosphorylation of eNOS at the Ser-1179 (Sessa, 2004) and as a positive
feedback mechanism, the activated NO signalling can induce Akt expression as a
downstream target of NO (Kawasaki et al. 2003). Thus it is possible that NO may
induce Akt expression to promote GMT driven cardiac reprogramming.

Canonical WNT3A gene expression was also up regulated during reprogramming
(Fig 5.8) raising the possibility that NO may promote canonical WNT signalling to
induce cardiac reprogramming. Supporting this hypothesis, inhibition of the WNT
signalling pathway by Dkk-1 showed attenuated cardiac specific TNN2 and ACTC
gene expression (Fig 5.8). Davidson et al reported that activation of canonical
WNT signalling through CHIR99021 or addition of WNT protein could significantly
induce ESC differentiation towards a cardiac phenotype (Davidson et al. 2012). In
addition Cao et al also reported that addition of the WNT activator CHIR99021
could increase human fibroblast reprogramming towards cardiomyocytes (Cao et
al. 2016). It could be proposed that activation of eNOS-NO signalling may
transduce signals through Akt and Wnt signaling to promote cardiac
reprogramming, however, the relationship between the Akt and Wnt signalling
pathways remained to be explored. It has been shown that canonical Wnt
signalling can act as an upstream mediator of the Akt, by which canonical Wnt
induces mTORC2 to activate Akt (Shimobayashi and Hall, 2014) and some reports
have shown that Akt can be activated upstream of Wnt signalling, in which Akt can
phosphorylate Pygo2, a Wnt co-activator to promote TCF/LEF activation in the
nucleus (Li et al. 2015).

Based on the data provided here, the NO enhancing approach can be applied to
different cell sources as demonstrated with increased cardiac reprogramming of
equine adipose stem cells (Fig 5.9). However, reprogrammed cells in this study did not show any functional features such as spontaneous beating or response to calcium transients even though the reprogrammed cells expressed mature markers such as TNN2 and ACTC (Fig 5.4). It is possible this may be due to an immature phenotype as there was no evidence of an organized sarcomere like structure in the reprogrammed cells (Fig 5.4 and Fig 5.9). Organization of sarcomere like structures (basic units of striated heart muscle) is important for the functionality of cardiomyocytes (Robeiro et al. 2015). In this study a short differentiation period of 14 days was used, which may also not be long enough to obtain functional cardiomyocytes as other investigations have reported that 1-2 months may be needed to produce beating cells (Zhou et al. 2016; Ieda et al. 2010). However, this short in vitro system might represent a valuable platform for increasing CLCs which may mature following transplantation in vivo where the in vivo environment might be more permissive for functional cardiomyocyte reprogramming (Song et al. 2012; Qian et al. 2012).

5.6. Conclusion

The use of cellular reprogramming to generate cardiomyocytes for therapeutic applications in humans remains a challenging goal, with many technical obstacles remaining and strategies to enhance conversion efficiency are needed such as incorporation of the eNOS-NO signalling pathway to augment transcription factor based reprogramming.
5.7. References

1. Beckman JS, Koppenol WH. Nitric oxide, superoxide and peroxynitrite: the good, the bad and the ugly. Am. J. Physiol. 1996; 271: C1424–C1437

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Chapter 6: Minicircle DNA-mediated endothelial nitric oxide synthase gene transfer enhances angiogenic responses of bone marrow-derived mesenchymal stem cells

Article published in *Stem Cell Research and Therapy* and attached in appendix 5 (pp 403-417)
6.1. Abstract

Non-viral-based gene modification of adult stem cells with endothelial nitric oxide synthase (eNOS) may enhance production of nitric oxide and promote angiogenesis. Nitric oxide (NO) derived from endothelial cells is a pleiotropic diffusible gas with positive effects on maintaining vascular tone and promoting wound healing and angiogenesis. Adult stem cells may enhance angiogenesis through expression of bioactive molecules, and their genetic modification to express eNOS may promote NO production and subsequent cellular responses. In this study, rat bone marrow-derived mesenchymal stem cells (rBMSCs) were transfected with a minicircle DNA vector expressing either green fluorescent protein (GFP) or eNOS. Transfected cells were analysed for eNOS expression and NO production and for their ability to form in vitro capillary tubules and cell migration. Transcriptional activity of angiogenesis-associated genes, CD31, VEGF-A, PDGFRα, FGF2, and FGFR2, were analysed by quantitative polymerase chain reaction. In results, minicircle vectors expressing GFP (MC-GFP) were used to transfect HEK293T cells and rBMSCs, and were compared to a larger parental vector (P-GFP). MC-GFP showed significantly higher transfection in HEK293T cells (55.51 ± 3.3 %) and in rBMSC (18.65 ± 1.05 %) compared to P-GFP in HEK293T cells (43.4 ± 4.9 %) and rBMSC (15.21 ± 0.22 %). MC-eNOS vectors showed higher transfection efficiency (21 ± 3 %) compared to P-eNOS (9 ± 1 %) and also generated higher NO levels. In vitro capillary tubule formation assays showed both MC-eNOS and P-eNOS gene-modified rBMSCs formed longer (14.66 ± 0.55 mm and 13.58 ± 0.68 mm, respectively) and a greater number of
tubules (56.33 ± 3.51 and 51 ± 4, respectively) compared to controls, which was reduced with the NOS inhibitor L-NAME. In an in vitro wound healing assay, MC-eNOS transfected cells showed greater migration which was also reversed by L-NAME treatment. Finally, gene expression analysis in MC-eNOS transfected cells showed significant upregulation of the endothelial-specific marker CD31 and enhanced expression of VEGFA and FGF-2 and their corresponding receptors PDGFRα and FGFR2, respectively. In summary, a novel eNOS-expressing minicircle vector can efficiently transfect rBMSCs and produce sufficient NO to enhance in vitro models of capillary formation and cell migration with an accompanying upregulation of CD31, angiogenic growth factor, and receptor gene expression.
6.2. Introduction

Development of safe and efficient systems for gene transfer is required for translation of gene-modified stem cells into therapeutic applications. Conventional plasmid DNA (pDNA)-based non-viral vectors contain bacterial sequences and transcriptional units that may contribute to an immune response against bacterial proteins expressed from cryptic upstream eukaryotic expression signals. Furthermore, changes in eukaryotic gene expression may be altered due to the antibiotic resistance marker and immune responses to bacterial CpG sequences (Kay et al. 2010). These prokaryotic DNA sequences present in pDNA vectors may lower their biocompatibility and safety. In clinical studies, un-methylated CpG motifs induced inflammatory responses (Zhang et al. 2010) and necrosis- or apoptosis-mediated cell death in target cells, resulting in short-lived transgene expression (Mitsui et al. 2009; Takahashi et al. 2012). Furthermore, during the intracellular trafficking of pDNA, the bacterial sequences of pDNA vectors are rapidly associated with histone proteins, packing the sequences into a dense heterochromatin structure. If these are spread into the adjacent transgene in the vector, the sequences can become inaccessible by transcription factors, leading to reduced transgene expression through silencing of the eukaryotic promoter (Riu et al. 2007). The removal of CpG islands by cloning out, or elimination of non-essential sequences, can reduce these undesirable responses but is time-consuming and tedious.

Minicircle (MC) pDNA technology consists of supercoiled DNA molecules for non-viral gene transfer, which has neither a bacterial origin of replication nor an
antibiotic resistance gene (Maniar et al. 2013). MCs can be generated in *E. coli* ZYCY10P3S2T by attachment sites (*attP* and *attB*), with specific recombination mediated by the phage ΦC31 integrase (Kay et al. 2010). As a result of this recombination event between *attP* and *attB* sites, MCs contain only a eukaryotic expression cassette and the *attR* fragments are formed but are devoid of bacterial backbone sequences. Absence of the bacterial backbone sequences leads to a size reduction in the MC relative to the parental pDNA which can enhance in vitro transfection efficiency (Dad et al. 2014) and in vivo gene delivery (Dietz et al. 2013; Osborn et al. 2011). Gene expression from non-viral episomal vectors may also enhance persistence of transgene expression without interrupting to the cellular genome (Broll et al. 2010).

Endothelial nitric oxide synthase (eNOS), also known as NOS3, is expressed in endothelial cells (Parathath et al. 2007), and is responsible for generating nitric oxide (NO) which plays an important role in vasculogenesis (Yu et al. 2005; Gomes et al. 2013). NO produced from endothelial cells is important for maintaining vascular integrity and may enhance vasculogenesis through fibroblast growth factor (FGF) signalling (Oladipupo et al. 2014). Vascular endothelial growth factor (VEGF) is also induced by the NO synthesis pathway (Sunshine et al. 2012) contributing to angiogenesis. eNOS knockout mice (*eNOS*−/−) display impaired vasculogenesis (Dai et al. 2010) and have also demonstrated diminished wound healing due to reduced VEGF-mediated migration of endothelial cells (Aicher et al., 2003) and bone marrow progenitor cells (Lu et al. 2011) to the sites of injury. eNOS based gene therapy approaches have shown restoration of impaired
angiogenesis in rats (Smith Jr et al. 2002; Tai et al. 2004) and promotion of re-endothelialisation (Sharif et al. 2008) in injured rabbits upon adenovirus-mediated eNOS gene transfer.

Similar to endothelial progenitor cells, mesenchymal stem cells (MSCs) also participate in post-natal angiogenesis (Bauth, 2011), and vascular pericytes, which are crucial for maintaining vascular integrity, share similar phenotypic features with MSCs (Caplan, 2008). Exogenously administered, MSCs form new capillaries and medium-sized arteries (Quevedo et al. 2009) which are important properties of tissue regeneration by MSCs (Williams et al. 2011). MSCs can differentiate into endothelial cells in vitro (Oswald et al. 2004) and contribute to neovascularisation, particularly during tissue ischaemia and tumour vascularisation (Sun et al. 2005). In MSCs, VEGF-A binds with platelet-derived growth factor receptor (PDGFR) to initiate VEGF-A/PDGFR signalling and drive vasculogenesis, as opposed to the VEGFR2 in endothelial cells, which is absent on MSCs (Ball et al. 2007). NO has been shown to upregulate PDGFRα receptor expression in rat mesangial cells (Beck et al. 2005), and the induction of tumour angiogenesis has been linked to the NO-induced Notch signalling pathway in PDGFR-activated mouse glioma cells (Charles et al. 2010). FGF2 signalling also enhances vasculogenesis through promotion of NO production (Straume et al. 2012; Murphy et al. 2001). eNOS is the only NOS isoform absent in MSCs (Gomes et al. 2013), and hence eNOS-based genetic modification of MSCs may enhance their therapeutic application. This study describes a novel non-viral MC vector to deliver the eNOS transgene to MSCs with higher transfection efficiency than regular plasmids. NO signalling in
the gene-modified MSC promotes capillary tube-like network formation and cell motility. Quantitative real time polymerase chain reaction (PCR) data revealed that MC-mediated eNOS gene transfer significantly upregulates endothelial-specific CD31 gene expression. Furthermore, NO up regulates the angiogenic responsive genes VEGF-A and FGF2 and expression of their corresponding receptors, PDGFRα and FGFR2.

6.3. Methods

6.3.1. Rat bone marrow-derived mesenchymal stem cell isolation

All experiments involving animals were approved by the Charles Sturt University animal ethics committee. MSCs were isolated from the bone marrow of 8–12 week old male Sprague–Dawley rats as previously described (McGinley et al. 2011, sections 2.2, chapter 2).

6.3.2. Tri-lineage differentiation of rBMSC

The ability of the isolated rat bone marrow-derived MSCs (rBMSCs) (Passage 6) to differentiate to adipogenic, osteogenic and chondrogenic lineages was investigated. To induce osteogenic differentiation, rBMSCs at 80–90 % confluency were incubated in osteogenic-defined medium (Dulbecco’s modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 10 mM beta-glycerol phosphate, 10 nM dexamethasone and 0.2 mM L-ascorbic acid 2-phosphate) for 11 days with medium changed twice a week, as described previously (Shih et al.
Cells were then fixed with 4 % paraformaldehyde and stained with Alizarin Red S (pH 4.1) as described previously (Fan et al. 2011).

To induce adipogenic differentiation, rBMSCs at 80–90% confluence were incubated in adipogenic-defined medium (DMEM supplemented with 10 % FBS, 10 μM indomethacin, 1 μM dexamethasone, 0.8 μM insulin, 0.5 mM rosiglitazone) (Liu et al. 2015) for 1 week with media changed twice. Adipogenic differentiation was assessed by 0.18 % Oil Red O staining after fixing the cells in 10 % neutral-buffered formalin (NBF) (Fan et al. 2011).

To induce chondrogenic differentiation, three-dimensional pellet cultures of rBMSCs (2.5 × 10⁵ cells) were formed by centrifugation at 500 × g in 10 ml conical-bottomed sterile tubes. The chondrogenic induction medium consisted of DMEM supplemented with 1 × ITS + 3 (Sigma), 1 × non-essential amino acids (Sigma), 10 ng/ml transforming growth factor β (TGF-β3; Peprotech), 100 nM dexamethasone, and 2 μM ascorbic acid (Sigma) (Ye et al. 2014). Pellet cultures were incubated in induction medium for 14 days with the medium changed every second day with the lids of the tube loosened to facilitate gas exchange. At day 14 the pellets were fixed in 10 % NBF for 24 h, and the three-dimensional tissues were processed and embedded in paraffin wax for microtome processing. To assess chondrogenic differentiation, embedded pellets were sectioned (5 μm slices) and stained with 1 % Alcian blue to visualise glycosaminoglycan accumulation.

The images for differentiated cells into all three lineages were captured by a colour camera (Nikon Digital Sight Ds-Fi2) attached to a Nikon Eclipse-Ti-U microscope (Nikon).
6.3.3. Production of minicircle plasmid DNA expressing eNOS

To construct an eNOS expressing minicircle vector, a codon optimized human eNOS cDNA sequence (3633 bp) was cloned into the minicircle parental plasmid consisting of expression cassette CMV–MCS–EF1α–GFP–SV40–PolyA (P-GFP) (System Biosciences, Mountain View, CA, USA). This cloning strategy allowed removal of the EF1α–GFP portion from the final construct (P-eNOS).

The minicircle DNA plasmids expressing eNOS and GFP were produced according to the manufacturer’s instructions (System Biosciences). Briefly, *E. coli* ZYCY10P3S2T cells were transformed with P-GFP and P-eNOS. Following this, single colonies were grown in 2 ml LB (Luria Bertani) media containing of 50 μg/ml kanamycin for 1 h at 30 °C with vigorous shaking at 200 rpm. Next, 50 μl of the starter culture was then used to inoculate 200 ml fresh terrific broth (TB; Sigma) in a 1 litre flask with 50 μg/ml kanamycin followed by incubation at 30 °C for 17 h with constant shaking at 200 rpm. Minicircle induction medium consisting of 200 ml LB broth, 8 ml 1 N NaOH and 200 μl 20 % L-arabinose was combined with the TB bacterial culture and incubated for a further 4 h at 30 °C with constant shaking at 200 rpm. Minicircle plasmid DNA (MC-eNOS and MC-GFP) was isolated using a Genomed Jetstar 2.0 midi kit according to the manufacturer’s instructions (Genomed, Germany) and treated with plasmid-safe ATP-dependent DNase (Epicentre, USA) to remove bacterial genomic DNA contamination. eNOS- and GFP-containing minicircles were designated as MC-eNOS and MC-GFP, respectively.
6.3.4. Cell culture and transfection

Human embryonic kidney (HEK293T) cells and rBMSCs were maintained in DMEM (Sigma) supplemented with 10 % (v/v) FBS (Sigma), 1 % (v/v) L-glutamate (Sigma) and 1 % (v/v) penicillin/streptomycin antibiotics mix (Sigma). Cells were transfected with the plasmids (P-GFP, MC-GFP, P-eNOS and MC-eNOS) using Lipofectamine 2000 reagent (Life technologies, USA) following the manufacturer’s instructions. GFP expression was assessed by fluorescence microscopy at 24 and 48 h after transfection, and flow cytometry analysis (Gallios Instrument, Beckmann).

6.3.5. Immunocytochemistry

Immunocytochemical detection of eNOS expression in P-eNOS and MC-eNOS transfected HEK293T and rBMSCs was performed as follows. Briefly, cells were fixed in 4 % paraformaldehyde for 20 min at room temperature, treated with 0.1% Triton-X100 in phosphate-buffered saline (PBS) for 10 min, and blocked in a 10 % FBS in PBS solution for 30 min at room temperature. This was followed by a 2 h incubation with a primary mouse monoclonal anti-eNOS antibody (1:100; BD Bioscience), and subsequently with an anti-mouse IgG secondary antibody conjugated with Alexa 488 (1:200; Cell Signalling Technology) for 1 h followed by DAPI (nuclear stain) and phalloidin-TRITC (cytoskeleton stain) (Sigma). eNOS positive cells were counted by fluorescence microscopy in five randomly selected fields per well in three independent experiments and 500–1000 cells were counted
in total and the percentage of eNOS positivity was calculated from the total nuclear stained cells.

### 6.3.6. Nitrite measurement

Nitrite released from P-eNOS and MC-eNOS transfected cells in cell supernatants was measured using the Griess reagent (Promega) following the manufacturer’s instructions (Standard curve (from 0.1 µM to 10 µM) was prepared). NO was also directly detected in transfected cells using a specific fluorescent NO indicator, 4,5-diaminofluorescein diacetate (DAF-2DA; Cayman chemicals, USA), as described previously (Gomes et al. 2013; Iwakiri et al. 2006). Cells were grown to confluence on a 12-well plate and incubated for 30 min with 1 µM DAF-2DA. Subsequently, cells were washed with fresh PBS and viewed by a fluorescence microscope.

### 6.3.7. In vitro angiogenesis

*In vitro* capillary formation was performed as described previously (Faulkner et al. 2014). Briefly, Geltrex™ (Life technologies) was thawed on ice overnight and applied evenly over each well (50 µl) of a 96-well plate and incubated for 30 min at 37 °C allowing polymerisation. Transfected rBMSCs or control cells were seeded at 20,000 cells per well and grown in 100 µl angiogenic induction medium (DMEM (Sigma), 1.5 % FBS, 1 % (v/v) L-glutamate (Sigma) and 1 % (v/v) penicillin/streptomycin (Sigma)) and incubated at 37 °C for 5 h. The capillary
network was fixed with 4 % paraformaldehyde and visualized by staining with DAPI and Phalloidin (Sigma). The efficiency of in vitro tubule formation was evaluated by measuring the number of nodes and length of the tubules as described previously (Gomes et al. 2013).

6.3.8. In vitro scratch wound healing assay

The effect of nitric oxide on cell migration was assessed using an in vitro scratch wound healing assay as described previously (Ye et al. 2014). Briefly, HEK293T cells and rBMSCs were transfected with P-eNOS, MC-eNOS, P-GFP and MC-GFP in 6-well tissue culture plates. Next, 48 h following the transfection when the cells reached 100 % confluence, scratch wounds were made using a sterile 200 μl pipette tip and the boundaries were marked. The cells were then cultured with 2 ml fresh DMEM supplemented with 10 % (v/v) FBS (Sigma), 1 % (v/v), L-glutamate (Sigma), and 1 % (v/v) penicillin/streptomycin (Sigma). Phase-contrast microscopy images were acquired at 0 and 1 h after scratches were created for rBMSCs and after 17 h for HEK293T cells. Cell migration was measured at the indicated times by measuring the distance from the initial boundary edge to the boundary of the migrating cells, followed by calculation of the percentage of wound closure as follows: percentage of wound closure = (distance from the boundary edge at 0 h – distance from the boundary edge at 1 h or 17 h) / (distance from the boundary edge at 0 h) × 100.
6.3.9. Gene expression by quantitative real time PCR

Total RNA from transfected and control cells was isolated using the PureZol reagent (BioRad) according to the manufacturer’s instructions and the concentration of isolated RNA was determined using a Nanodrop spectrophotometer (Thermo Scientific) following treatment with RQ1 RNase free DNase (Promega) to remove contaminating DNA. Then, cDNA was synthesized with 1 μg RNA using a High Capacity Reverse Transcription Kit (Life technologies). The quantitative real time PCR assays were performed on a BioRad CFX96 Real-Time system (BioRad) using the SsoFast EvaGreen Supermix (BioRad). Primers used for target amplification are described in Table 6.1. Assays were performed in triplicate, and target mRNA expression was normalized to rat GAPDH mRNA levels using the ΔΔCt method.
Table 6.1. Primers used in this study

<table>
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<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
<th>Expected size</th>
<th>Accession number</th>
</tr>
</thead>
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<td>GGTGGACATCTTCCAGGAGT</td>
<td>TGATCTGCATGGTGATGTTG</td>
<td>146</td>
<td>NM_001317043</td>
</tr>
<tr>
<td>Fgf2</td>
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<td>129</td>
<td>NM_019305</td>
</tr>
<tr>
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</tr>
<tr>
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<td>134</td>
<td>EF143338</td>
</tr>
<tr>
<td>Cd31</td>
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<td>GTCTTACCCAGCCTTTCTC</td>
<td>104</td>
<td>NM_001107202</td>
</tr>
<tr>
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<td>TTTGAGGGTGCGAAGCTT</td>
<td>252</td>
<td>NM_017008.4</td>
</tr>
</tbody>
</table>

6.3.10. Western blot analysis

Transfected and control cells were washed with ice-cold PBS (Sigma) twice, and lysates were prepared by homogenization of cells in RIPA buffer (Sigma), following mixing with 4 × NuPAGE LDS sample buffer (Life technologies) and lysed by heating for 10 min at 70 °C. Total proteins were separated by 4–12 % Bis-Tris NuPAGE (Novex, Life technologies) and transferred to PVDF membrane (Millipore). After blocking with odyssey blocking buffer (LI-COR) for 30 min at room temperature, the membrane was incubated with primary antibodies specific to eNOS (1:1000 dilution) and β-actin (LI-COR; 1:1000 dilution) overnight at 4 °C. The membrane was washed with 0.1 % tween in PBS three times for 10 min each, incubated with donkey anti-rabbit IgG (H&L) (Alexa Fluor® 680) secondary antibody (Life technologies; 1:20,000) at room temperature for 1 h, and antibody-
bound proteins were visualized by fluorescence detection with a LI-COR odyssey system.

6.3.11. Statistical analysis

All experiments were performed in triplicate and at least three times. Data are presented as mean ± SEM. The statistical significances were determined by one-way analysis of variance (ANOVA) followed by post hoc. All tests were performed using the statistical software GraphPad Prism 6 (GraphPad, CA, USA). $p<0.05$ was considered statistically significant.
6.4. Results

6.4.1. Characterisation of Rat BMSCs

rBMSCs were isolated from adult Sprague–Dawley rats as previously described (McGinley et al. 2011), and plastic adherent rBMSCs displayed typical fibroblastoid morphology (Fig. 6.1a) (Xu et al., 2012). Tri-lineage differentiation of the rBMSCs was performed in the appropriate media to osteoblasts as demonstrated by Alizarin Red S staining of mineralised extracellular matrix (Fig. 6.1b), to chondrocytes by Alcian Blue staining of proteoglycans in three-dimensional pellet cultures (Fig. 6.1c) and to adipocytes as shown by Oil Red O staining of lipid vesicles (Fig. 6.1d).
Figure 6.1. Characterization of rBMSCs. Tri-lineage differentiation of rBMSC was performed *in vitro*. 

(a) Undifferentiated rBMSC. 

(b) Alizarin red S staining of cells cultured for 14 days in osteogenic induction medium. 

(c) Alcian blue staining and toluidine blue staining of cells cultured for 14 days in chondrogenic induction medium. 

(d) Oil red O staining of cells cultured for 7 days in adipogenic induction medium. Scale bar=100 μm.
6.4.2. Transfection of P-GFP and MC-GFP vectors

The GFP expressing minicircle vector (MC-GFP) was produced from the parental plasmid (P-GFP) as described in the manufacturer’s instructions (Systems Bioscience). An approximate 4-kb reduction in plasmid size was observed following minicircle induction using L-arabinose (Fig. 6.2a). HEK293T cells and rBMSCs were transfected with a range of plasmid DNA concentrations (1 μg, 0.5 μg, 0.25 μg, 0.125 μg, 0.0625 μg,). After 48 h post-transfection, the cells were visualised by fluorescence microscopy and analysed by flow cytometry to estimate the percentage of GFP-expressing (GFP+) cells (Fig. 6.2b). The optimum plasmid DNA concentration for transection was 0.5 μg which showed highest transfection efficiency for both P-GFP and MC-GFP in both HEK293T (Fig.6.2c) and rBMSC (Fig. 6.2d) cell types. Transfection of HEK293T cells with MC-GFP plasmid resulted in a significantly higher number of GFP+ cells (55.51 ± 3.3 %) compared to P-GFP (43.4 ± 4.9 %). A similar trend was seen in rBMSCs, with MC-GFP resulting in higher transfection efficiency (18.65 ± 1.05 %) compared to P-GFP (15.21 ± 0.22 %).
Figure 6.2. Gene delivery efficiency of P-GFP and MC-GFP DNA vectors. a Gel electrophoresis of P-GFP and MC-GFP plasmids following plasmid purification and enzyme digestion. b Fluorescence microscopy of transfected HEK293T cells and rBMSCs with P-GFP and MC-GFP with a range of plasmid concentrations and quantitation by flow cytometry of transfection efficiencies for (c) HEK293T cells and (d) rBMSCs. *p<0.05 vs. P-GFP. One way ANOVA followed by post hoc. N=3. Scale bar=100 μm
6.4.3. Generation of eNOS minicircle vector

To generate an eNOS minicircle expression plasmid vector, a codon optimized cDNA of human eNOS (3633 bp) was synthesised (Geneart) and sub-cloned into the parental plasmid P-GFP (CMV-MCS-EF1-GFP-SV40PolyA) (System Biosciences, Mountain View, CA, USA) at the BamHI and SalI restriction sites in the multiple cloning sites downstream to the CMV promoter resulting in removal of the EF1α promoter and eGFP coding sequence (Fig. 6.3a). The eNOS minicircle vector was constructed as described above for the MC-GFP vector. The cloning was confirmed by double digestion of the parental plasmid encoding eNOS (P-eNOS) with BamHI and SalI yielding a fragment of ~3.7 kb (Fig. 6.3b). A reduction of the P-eNOS vector size was also observed after the production of MC-eNOS, to approximately 5 kb (Fig. 6.3c).
Figure 6.3. Construction of eNOS expressing minicircle DNA vector. **a** Schematic representation of in vitro production of MC-eNOS vector. **b** Confirmation of cloning of eNOS gene into P-eNOS and MC-eNOS vectors by restriction enzyme digestion analysis. **c** P-eNOS and MC-eNOS gel electrophoresis following minicircle plasmid purification.
6.4.4. Transfection of P-eNOS and MC-eNOS vectors

Transfection of HEK293T cells with P-eNOS and MC-eNOS was assessed by immunofluorescence staining (Fig. 6.4a) and western blot analysis (Fig. 6.4b), using an eNOS-specific monoclonal antibody (BD bioscience). Nitric oxide production from transfected cells was measured by the production of nitrite at 24 and 48 h post-transfection and in un-transfected HEK293T cells (Fig. 6.4c). Both P-eNOS and MC-eNOS transfected HEK293T cells showed significantly higher nitrite accumulation in cell culture media (at both 24 h and 48 h) compared to P-GFP, MC-GFP transfected cells and un-transfected HEK293T controls. At 24 h post-transfection, HEK293T cells transfected with P-eNOS and MC-eNOS resulted in 3.8 ± 0.2 μM and 4.46 ± 0.12 μM nitrite concentrations, respectively (Fig. 6.4c). The NO production increased significantly at 48 h post-transfection, resulting in 4.18 ± 0.12 μM and 5.06 ± 0.13 μM for P-eNOS and MC-eNOS, respectively. Furthermore, detection of nitric oxide produced from transfected cells was also confirmed by DAF-2DA staining in live cells. Both P-eNOS and MC-eNOS transfected HEK293T cells emitted a strong green fluorescence signal compared to no fluorescence in un-transfected cells (Fig. 6.4d).
Figure 6.4. Expression of eNOS and NO production in transfected HEK293T cells. 

a) Fluorescence microscopy of transfected HEK293T cells with P-eNOS and MC-eNOS with 0.5 μg plasmid DNA (Dapi, nuclear staining, TRITC, cytoskeleton, GFP, eNOS expression).

b) Detection of eNOS protein expression in transfected HEK293T by western blot analysis.

c) Nitrite levels in HEK293T cells at 24 h and 48 h post-transfection with P-eNOS and MC-eNOS plasmids using the griess assay, and 
d) detection of nitric oxide production in living cells following P-eNOS and MC-eNOS transfection and non-transfected control by DAF-2 fluorescence. *p<0.05 and **p<0.05 vs. MC-GFP, P-GFP, and HEK293T. One way ANOVA followed by post hoc. N=3. Scale bar=100 μm.
6.4.5. eNOS gene transfer to rBMSCs

Transfection of P-eNOS and MC-eNOS vectors into rBMSCs was confirmed by immunostaining (Fig. 6.5a), and western blot analysis (Fig. 6.5c) with an eNOS-specific monoclonal antibody (BD bioscience). Both the assays confirmed that no endogenous eNOS expression was seen in un-transfected rBMSCs (Fig. 6.5a and c). Significantly higher transfection efficiency for MC-eNOS (21 ± 3 %) compared to P-eNOS (9 ± 3 %) (Fig. 6.5b) was observed which resulted in higher nitrite levels for MC-eNOS transfected rBMSCs (1.93 ± 0.06 μM) than P-eNOS (1.78 ± 0.1 μM) (Fig. 6.5d) compared to controls after 24 h of transfection. Nitrite levels increased further in MC-eNOS transfected rBMSCs (2.20 ± 0.08 μM) compared to P-eNOS at 48 h post-transfection (1.84 ± 0.1 μM) (Fig. 5d). NO synthesis in transfected rBMSCs was also demonstrated DAF-2DA staining in both P-eNOS and MC-eNOS transfected rBMSCs (Fig. 6.5e).
Figure 6.5. Expression of eNOS and NO production in transfected rBMSCs. **a** Fluorescence microscopy (Dapi; nuclear staining, TRITC; cytoskeleton staining, GFP; eNOS expression with eNOS specific antibody) of transfected rBMSCs with P-eNOS and MC-eNOS with 0.5 μg plasmid DNA. **b** Transfection efficiency of MC-eNOS and P-eNOS. **c** Detection of eNOS protein expression in transfected rBMSC by western blot analysis. **d** Nitrite levels in rBMSC cells at 24 and 48 h post-transfection with P-eNOS and MC-eNOS plasmids using the griess assay. **e** Detection of nitric oxide production in living cells following P-eNOS and MC-eNOS transfection by DAF-2 fluorescence. *p<0.05 vs. P-eNOS; *p<0.05 and **p<0.05 vs. MC-GFP, P-GFP, and rBMSC. One way ANOVA followed by post hoc. N=3. Scale bar=100 μm.
6.4.6. eNOS gene delivery enhances *in vitro* capillary tubule formation.

Rat BMSCs were transfected with 0.5 μg P-eNOS, MC-eNOS, P-GFP, and MC-GFP. Un-transfected rBMSCs were used as a control. Transfected cells were then plated on a 96-well cell culture plate coated with an extracellular matrix (Geltrex). Both MC-eNOS and P-eNOS transfected rBMSCs formed significantly longer (14.66 ± 0.55 mm and 13.58 ± 0.68 mm, respectively) tubules and a greater number of tubules (56.33 ± 3.51 and 51 ± 4, respectively) compared to rBMSCs transfected with P-GFP, MC-GFP and non-transfected cells (Fig. 6.6).

To confirm that capillary-like tubule formation was NO-mediated, eNOS transfected rBMSCs were treated with 2 mM of the nitric oxide synthase inhibitor, L-N\(^{G}\)-nitroarginine methyl ester (L-NAME). L-NAME treatment resulted in a significant impairment of the tubule network, in terms of length (7.33 ± 1.03 mm and 7.06 ± 0.88 mm for MC-eNOS and P-eNOS, respectively) and tubule number (24 ± 4 and 24 ± 2 for MC-eNOS and P-eNOS, respectively) compared to untreated cells (Fig. 6.6).
Figure 6.6. *In vitro* tubule formation in eNOS transfected rBMSCs. **a** Capillary tubule formation in rBMSCs transfected with P-eNOS and MC-eNOS and cytoskeletal staining by Phalloidin TRITC; treatment with the NO inhibitor L-NAME reduces capillary formation. **b** Quantitation of tubule number. **c** Measurement of tubule length. *p<0.05 and **p<0.05 vs. MC-eNOS (L-NAME), P-eNOS (L-NAME), MC-GFP, P-GFP, and rBMSC. One way ANOVA followed by post hoc. N=3.*
6.4.7. Nitric oxide promotes \textit{in vitro} cell migration

Using the scratch wound healing assay (Ye et al., 2014), migration of eNOS transfected rBMSCs was assessed. Transfection of P-eNOS and MC-eNOS enhanced cell migration compared to P-GFP, MC-GFP and un-transfected rBMSCs (MC-eNOS, 44.05 ± 0.81 %; P-eNOS, 43.13 ± 3.45 %; MC-GFP, 10.43 ± 2.63 %; P-GFP, 11.39 ± 3.03 %; and rMSC, 9.46 ± 4.13 %) (Fig. 6.7a and c). However, cell migration rates between P-eNOS and MC-eNOS were not significantly different. Inhibition of NO production by treatment with 2 mM \textit{l}-NAME significantly diminished the cell migration rates of both MC-eNOS and P-eNOS transfected cells (12.18 ± 1.67 % and 15.59 ± 4.69 %, respectively) (Fig. 6.7a and c). Cell migration rates were not significantly different among MC-GFP, P-GFP and un-transfected cells (Fig. 6.7a and c). A similar phenomenon was observed with HEK293T cells (Fig. 6.7b and d).
Figure 6.7. *In vitro* cell scratch assay of eNOS transfected rBMSCs and HEK293T cells. Phase-contrast microscopy images of transfected and control cell migration at (a) 0 and 1 h for rBMSCs and (b) 0 and 17 h for HEK293T post-cell scratch and effect of the NO inhibitor L-NAME. Percentage of cell migration (c) at 1 h for rBMSCs and (d) 17 h for HEK293T post-cell scratch and effect of the NO inhibitor L-NAME. *p<0.05 and **p<0.05 vs. MC-eNOS (L-NAME), P-eNOS (L-NAME), MC-GFP, P-GFP, rBMSC and HEK293T. One way ANOVA followed by post hoc. N=3.
6.4.8. MC-eNOS gene transfer to rBMSCs induces endothelial Cd31 gene expression

A significant increase in Cd31 mRNA expression by 0.42-fold was seen in P-eNOS transfected cells compared to P-GFP, MC-GFP and un-transfected control, suggesting that eNOS gene transfer may promote endothelial differentiation of rBMSCs (Fig. 6.8). Interestingly, minicircle-mediated eNOS (MC-eNOS) gene transfer showed a highly significant increase in Cd31 mRNA expression by 1.8-fold compared to P-eNOS, P-GFP, MC-GFP, and un-transfected control (Fig. 6.8). Treatment with 2 mM L-NAME abolished the Cd31 expression (Fig. 6.8), suggesting that expression of endothelial Cd31 in rBMSCs through eNOS gene transfer is NO-mediated.
Figure 6.8. Nitric oxide promotes *Cd31* gene expression in eNOS transfected rBMSCs. Relative mRNA expression of endothelial-specific *Cd31* was upregulated in MC-eNOS and P-eNOS transfected rBMSCs as assessed by quantitative real time PCR. *p<0.05 and **p<0.05 vs. MC-eNOS (L-NAME), P-eNOS (L-NAME), MC-GFP, P-GFP, and rBMSC. One way ANOVA followed by post hoc. N=3.
6.4.9. NO modulates Vegf-a/Pdgfr and Fgf2/Fgfr2 signalling pathways in eNOS transfected rBMSCs

Expression of two key genes, Vegf-a and Fgf2, which are involved in angiogenesis and cell migration were examined by quantitative real time PCR. Up regulation of both Vegf-a by 1.19-fold and 1.0-fold in MC-eNOS and P-eNOS modified rBMSCs, respectively (Fig. 6.9), and Fgf2 by 1.08-fold in MC-eNOS and 0.74-fold in P-eNOS delivered rBMSCs (Fig. 6.9b), compared to P-GFP, MC-GFP delivered rBMSCs and un-transfected rBMSCs. Treatment with 2 mM L-NAME reduced both Vegf-a (Fig. 6.9a) and Fgf2 (Fig. 6.9b) expression in P-eNOS and MC-eNOS transfected cells. Furthermore, delivery of P-GFP and MC-GFP did not affect the Vegf-a and Fgf2 expression compared to control rBMSCs (Fig. 6.9a and 6.9b). Next, the effect of NO on the expression of Pdgfra and Fgfr2 receptors was examined as they are corresponding receptors of Vegf-a and Fgf2. Expression of Pdgfra was increased by 1.82-fold and 1.56-fold in MC-eNOS and P-eNOS transfected rBMSCs, respectively (Fig. 6.9c), and Fgfr2 receptor expression was increased by 1.46-fold in MC-eNOS and 1.14-fold in P-eNOS delivered rBMSCs (Fig. 6.9d), compared to P-GFP, MC-GFP delivered rBMSCs and un-transfected rBMSCs. Treatment with 2 mM L-NAME abolished both the Pdgfra (Fig. 6.9c) and Fgfr2 (Fig. 6.9d) expression in P-eNOS and MC-eNOS transfected cells. Furthermore, neither Pdgfra nor Fgfr2 receptor expression were affected by the delivery of P-GFP and MC-GFP compared to control rBMSCs (Fig. 6.9c and d).
Figure 6.9 Nitric oxide modulates Vegf-a/Pdgfra and Fgf2/Fgfr2 gene expression. Relative mRNA expression of the angiogenesis-related genes (a) Vegf-a and (b) Fgf2 and their corresponding receptors, (c) Pdgfra and (d) Fgfr2 were upregulated in MC-eNOS and P-eNOS transfected rBMSCs as assessed by quantitative real time PCR. *p<0.05 and **p<0.05 vs. MC-eNOS (L-NAME), P-eNOS (L-NAME), MC-GFP, P-GFP, and rBMSC. One way ANOVA followed by post hoc. N=3.
6.5. Discussion

Minicircle vectors are supercoiled DNA molecules that are devoid of bacterial backbone sequences such as a bacterial origin of replication, antibiotic resistance gene and CpG motifs (Jia et al. 2010), and primarily consist of a eukaryotic expression cassette (Maniar et al. 2013) Compared to conventional plasmid DNA, minicircle vectors benefit from higher transfection efficiencies and longer transgene expression, possibly attributed to a lower activation of gene silencing mechanisms (Chen et al. 2003).

In this study, minicircles expressing GFP exhibited higher in vitro gene transfer efficiency than the parental plasmid to both HEK293T cells and rBMSCs (Fig. 6.3). As expected transfection efficiency was higher in the transformed cell line (HEK293T) compared to primary rBMSCs. eNOS expressing minicircles also showed higher gene transfer efficiency than P-eNOS (Fig. 6.5b). This higher gene transfer efficiency may also account for the significantly increased level of NO synthesis by MC-eNOS compared to P-eNOS (Figs. 6.4 and 6.5). I reasoned that this high level of NO synthesis from MC-eNOS transfected rBMSCs may be attributed to the removal of other plasmid sequences, which can affect gene expression (Chen et al. 2003), and the smaller size of the minicircle may also provide a more efficient route to the nucleus for transcription. This process involves several steps, including cellular entry of DNA through the cell membrane, DNA diffusion into the cytoplasm, and DNA entry to the nucleus (Darquet et al. 1999). Importantly, the DNA diffusion step depends on the physicochemical properties of DNA such as its diffusion coefficient, which is inversely proportional to its...
molecular weight (Shen et al. 2006; Speit et al. 2014). Endocytosis is a major route for entry of DNA–cationic lipid complexes through the cell membrane in vitro (Le et al. 2011) which takes place following specific interactions between DNA and caveolae (Rejman et al. 2005). This mechanism is also limited by particle size, where larger DNA–cationic lipid complexes are not efficiently taken up by the endocytosis (Rejman et al. 2005). DNA uptake and transfer to the nucleus via the nuclear membrane results in successful gene transfer (Zabner et al. 1995). Minicircle plasmid vector may overcome these cellular obstacles more efficiently and, combined with a lack of bacterial backbone sequences, reduced promoter methylation (Hong et al. 2001) may also contribute to the higher levels of gene transfer compared to larger parental plasmids.

Angiogenesis is a complex process involving endothelial cell proliferation and migration, remodelling of extracellular matrix, and tubular structure formation. These processes are tightly regulated by the actions of angiogenic cytokines such as VEGF-A and FGF (Straume et al. 2012). Angiogenesis also requires endothelial cell-to-cell, and cell-to-matrix interactions, which are mediated by various cell adhesion molecules (Dejana et al. 1996). eNOS plays a key role in angiogenesis mediated by substance P, a potent endothelium-dependent vasodilator (NO releaser) (Ziche et al. 1994). It has also been demonstrated that eNOS-KO (knockout) mice show impaired angiogenesis (Murohara et al. 1998).

NO has been shown to play an important role in angiogenesis both in vitro and in vivo, and furthermore NO also contributes to endothelial cell migration in vitro (Murohara et al. 1998). The data from this study showed that, eNOS gene transfer...
by MC vector remarkably promoted endothelial-specific *Cd31* gene expression (Fig. 6.8), contributing to the capillary-like tubule network formation by rBMSCs (Fig. 6.6) and enhanced cell motility as evident by *in vitro* wound healing assay (Fig. 6.7). Noteworthy, in these assays, *Cd31* mRNA expression, tubule formation and cell migration in transfected cells were significantly abrogated by L-NAME treatment, suggesting NO plays a major role in enhancing endothelial characteristics in rBMSCs. Collectively, the data from this study may suggest that MC-mediated eNOS gene transfer may contribute to the reprogramming of adults stem cells into endothelial cells, which may be used in cell therapy applications involving vascular repair. Interestingly, Gomes and co-workers demonstrated that MSCs from S-nitrosoglutathione reductase (GSNOR)-deficient mice, where NO is produced mainly from iNOS (NOS2) rather than eNOS, exhibited attenuated vasculogenesis both *in vitro* and *in vivo* (Gomes et al. 2013). Furthermore, they revealed that pharmacological inhibition of NO in GSNOR−/− MSCs, or genetic reduction of NO production in the NOS2−/−, enhanced vasculogenesis by MSCs than that for HUVECs, where NO synthesis is driven by eNOS enhanced vascular tube formation. MSCs have not been shown to express endogenous eNOS, unlike endothelial cells (Kuhlencordt et al. 2004), and have been shown to participate in pro-angiogenic signalling (Huang et al. 2003). Additionally, eNOS plays an important role in endothelial cell-mediated postnatal angiogenesis and vascular tone (Beigi et al. 2012; Lima et al. 2009).

NO may contribute to angiogenesis through VEGF and FGF signalling through an angiogenic switch which is preceded by a local increase in VEGF-A and FGF
(Straume et al. 2012). Nitric oxide can mediate the production of VEGF-A in human adipose-derived stem cells (Bassaneze et al. 2010) and NO and FGF2 have also been shown to enhance angiogenesis in mouse embryonic stem cells (Sauer et al. 2013). Furthermore, FGF2 has been shown to induce eNOS expression (Murphy et al. 2001). The data from this study propose that NO signalling through VEGF-A/PDGFRα and FGF2/FGFR2 pathways may directly promote rBMSC vasculogenesis (Fig. 6.9). Results from this study showed that eNOS transfected rBMSCs express increased levels of Vegf-a and Fgf2 (Fig. 6.9) and their corresponding receptors Pdgfra, and Fgfr2, respectively (Fig. 6.9). It is noteworthy that MC-eNOS vector transfection was associated with a significantly higher Fgf2 expression compared to the P-eNOS vector. Interestingly, treatment with L-NAME diminished the Vegf-a, Pdgfra, Fgf2 and Fgfr2 expression levels (Fig. 6.9) which were observed as being linked to impaired capillary tube-like network formation (Fig. 6.6). It has been shown that Vegf-a contributes to differentiation of MSCs to endothelial-like cells when co-cultured with endothelial cells expressing eNOS and this process is inhibited by Vegf-a antiserum (Wu et al. 2005).

Angiogenesis is also associated with endothelial cell migration and proliferation (Carmeliet, 2003). Results from this study show that eNOS gene transfer into HEK293T and rBMSCs (Fig. 6.7) can increase cell motility compared to controls, and the effect is diminished by L-NAME treatment, suggesting that NO plays a role in regulating rBMSC cell migration (Fig. 6.7) which has been previously demonstrated for endothelial cell migration (Eller-Borges et al. 2015). Together, these findings show that genetic manipulation of MSCs to enhance bioavailable
NO may upregulate VEGF-A/PDGFRα and FGF2/FGFR2 signalling pathways to promote angiogenesis (Fig. 6.10).

6.6. Conclusions

In summary, this study demonstrates that NO derived from a minicircle DNA vector expressing eNOS exerts a positive effect on rBMSCs by promoting *in vitro* capillary tubule formation and cell migration and significant increases in angiogenesis-related gene expression. Use of MC-eNOS-based vectors may represent an efficient approach to gene therapy applications where enhancing NO bioavailability is beneficial.
Figure. 6.10 Proposed molecular mechanism underlying the NO mediated angiogenic responses by MSCs. Fgfr2: fibroblast growth factor 2 receptor. NO: nitric oxide, Pdgfr: platelet-derived growth factor receptor, Vegf: vascular endothelial growth factor.
6.7. References


Chapter 7: Alanine scanning and mutagenesis of caveolin-1 amino acid residues within the scaffolding sub-domain-A
7.1. Abstract

Nitric oxide (NO) plays an important role in a variety of physiological processes including, bone homeostasis, neo-angiogenesis, and cardiovascular development. NO is synthesized by nitric oxide synthase and one of which is endothelial NO synthase (eNOS), and which is negatively regulated by caveolin-1 (CAV-1), the major structural protein of the plasma membrane caveolae. Within the structure, the CAV-1 scaffolding domain (CSD, amino acids 82-101 of CAV-1) has been shown to interact with eNOS and inhibit enzyme activity. Within the CSD, three subdomains, CAV-A, CAV-B and CAV-C have been identified and CAV-A has been suggested to be important for CSD solubility. The current study was designed to analyze a series of new mutations within the CAV-A subdomain which may theoretically increase the solubility of CSD together with a previously characterized CAV-1F92A mutation in terms of nitric oxide production. An Alanine scanning approach was employed together with a bioinformatics analysis of the alanine substituted mutants of CAV-A to reveal a potential Ile-84 substitution to alanine (I84A) which may increase solubility of the CSD. A further four mutants were designed to complement F92A (CAV-1F92A-D82A, CAV-1F92A-G83A, CAV-1F92A-I84A, and CAV-1I84A), and generated by site directed mutagenesis and over expressed with eNOS in HEK293T cells using lentiviral vector technology and NO production was measured by the greiss assay. Compared to the original F92A mutation of CAV-1, no significant increased NO production was observed in HEK293T\textsuperscript{eNOS+CAV-1F92A-I84A} cells compared with CAV-1F92A and eNOS transduced cells.
Caveolin-1 is a 22-kDa membrane protein important for the formation of plasma membrane caveolae (Bai et al. 2014, Panic et al. 2017; Forrester et al. 2017). Caveolin-1 has been shown to be expressed in a variety of cell types such as endothelial cells (Kronstein et al. 2012; Pavlides et al. 2014), fibroblasts (Shen et al. 2015) and mesenchymal stem cells (Baker et al. 2015) and has been shown to play an important role in intracellular signalling via interaction with other proteins (Lee et al. 2015; Schönle et al. 2016). Caveolin-1 comprises of 178 amino acid residues and the caveolin-1 scaffolding domain (CSD, residues D82–R101) has been shown to interact with other client proteins (Couet et al. 1997), including endothelial nitric oxide synthase (eNOS) (Bernatchez et al. 2005).

eNOS is a well characterized cellular target of CAV-1 and the latter has been shown to inhibit eNOS activation and subsequent NO production (Bernatchez et al. 2005; Chen et al. 2012; Sharma et al. 2015; Forrester et al. 2017). CAV-1 knockout mice have also been shown to have increased endothelium derived NO synthesis (Razani et al. 2001; Mao et al. 2012) suggesting an inhibitory interaction of CAV-1 with eNOS. In addition, delivery of cell permeable CAV-1 peptides has been shown to block eNOS derived NO synthesis (Bernatchez et al. 2005; Bernatchez et al. 2011). Modification of caveolin-1 to include a substitution of phenylalanine (F) to alanine (A) at amino acid position 92 (F92A) could increase eNOS derived NO synthesis while still interacting with eNOS and furthermore delivery of this peptide containing the F92A mutation could increase eNOS derived NO synthesis and also rescued the impaired angiogenesis in mice (Bernatchez et
al. 2011). Recently, CAV-1<sup>F92A</sup> knock in transgenic mice have been generated demonstrating increased NO production (Kraehling et al. 2016), providing further evidence that the F92A mutation within the CAV-B subdomain can enhance eNOS derived NO synthesis.

CAV-B subdomain contains the hydrophobic sequences 89<sup>FTTFTVT</sup><sup>95</sup> and the CAV-A subdomain is considered to be a hydrophilic region (Bernatchez et al. 2005), and a detailed characterization of each the residues in the CAV-A subdomain in terms of hydrophilic properties remains to be investigated. CSD mediated direct protein-protein interaction has been shown to involve a signature peptide sequence of client proteins termed the caveolin binding motif (CBM) (Couet et al. 1997; Oka et al. 1997). Couet et al (1997) have originally reported these consensus CBMs in CSD interacting proteins through screening of a phage display peptide library and they have shown that, CBMs are composed of aromatic residues (ΩxΩxxxxΩ or ΩxxxxΩxxΩ or the combined sequence of ΩxΩxxxxΩxxΩ (Ω is a Phe, Tyr or Trp residue and X can be any amino acid) and CSD mediated CAV-1 interaction with these CBMs has been shown to play an inhibitory role on its client proteins (Okamoto et al. 1998; Bernatchez et al. 2005).

Since CBM consensus sequences are rich in hydrophobic aromatic side chains, it has been demonstrated that eNOS CBM interaction motifs in the CSD are located within CAV-B and C subdomains (90<sup>TTFTVT</sup>99<sup>KYWF</sup>) which are also composed of hydrophobic aromatic residues (Trane et al. 2014). Interestingly, substitution of eNOS CBM aromatic (hydrophobic) residues to alanine has been shown to prevent
inhibition by CAV-1 suggesting the disruption of CSD-CBM interaction when aromatic hydrophobic residues are removed (Garcia-Cardena et al. 1997).

In this study, amino acid residues of CAV-A subdomain which are critical for the solubility of CSD were investigated using an alanine scanning approach together with an in silico analysis which predicted that replacement of isoleucine (Ile) at amino acid position 84 (I84) with alanine (I84A) presented the lowest hydrophobicity among the alanine substituted CAV-A mutants. This suggested that incorporation of the I84A mutation together with the F92A mutation within the CAV-1 backbone may increase eNOS derived NO production. Next, CAV-1I84A mutant alone and double mutant with F92A (CAV-1F92A-I84A) were constructed and as controls two other alanine substituted double mutants at the amino acid positions 82 (D82A), and 83 (G83A) with F92A (CAV-1F92A-D82A, and CAV-1F92A-G83A respectively) were constructed by site directed mutagenesis. Surprisingly, there was not any significant increase in NO synthesis observed in HEK293T cells transduced with lentiviral vectors expressing CAV-1F92A-I84A and eNOS (HEK293TeNOS+CAV-1F92A-I84A) compared to HEK293TeNOS+CAV-1F92A even though increased solubility was predicted when I84 was mutated into A84 (I84A) in the bioinformatics analysis. Taken together, these data may identify that a single mutation of CAV-A subdomain may not have any significant additive effect on F92A mediated eNOS derived NO production.
7.3. Materials and methods

7.3.1. Cell culture, bacterial strains and growth conditions

Plasmids used in this work are listed in Table 7.1. *E. coli* Top10 cells were grown aerobically at 37°C in Luria-Bertani (LB) medium and ampicillin was added at the final concentration of 100 µg/ml. Human embryonic kidney 293T cells (HEK293T) (ATCC, Manassas, VA) were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10 % (v/v) fetal bovine serum (Lonza), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Growth medium) at 37°C and 5 % CO₂, and maintained.

7.3.2. Site-directed mutagenesis for generation of recombinant CAV-1 mutants

All oligonucleotides were synthesized from Sigma-Aldrich. Restriction and DNA modifying enzymes were purchased from New England Biolabs (NEB, MA, USA).

A 534-bp fragment of human caveolin-1 in which phenylalanine (F) at the amino acid position 92 was replaced with alanine (A) was synthesized and cloned into the pMA-T plasmid backbone (Geneart ,Supplementary figure 7.S1) and used as a template for site directed mutagenesis to generate double mutants carrying both the F92A and novel CAV-A mutations. Site directed mutagenesis was performed using PfuUltra High-Fidelity DNA Polymerase (NEB, MA, USA) PCR and DPN-I digestion following a standard protocol (Zheng et al. 2004; Liu and Naismith, 2008). Briefly, the total mutagenesis PCR reaction mixture volume of 50 µl
consisted of 10 µl of reaction buffer (5X), 1 µl dNTP mix from a 10 mM stock, 1 µl PfuUltra High-Fidelity DNA Polymerase, 50 ng plasmid DNA (pMA-T-CAV-1^{F92A}) and 1 µl each of the forward and reverse primers from a 100 µM stock (primers sequences used for each mutation are listed in Table 7.1) followed by total volume adjustment to 50 µl using deionized H2O. The reaction mixture was then heated to 95°C for 30 s followed by 16 cycles of 95°C for 30 s, 55°C for 1 min and 68°C for 5 min. The mixture was then incubated at 37°C for 1 h with DPN-I restriction enzyme (NEB, MA, USA) to digest the parental DNA prior to transformation into E. coli Top10 super competent cells. Successful mutagenesis was verified by DNA sequencing.

Both I84A mutant (CAV-1^{F92A-I84A} and CAV-1^{I84A}) were synthesized with BamH1 and SalI restriction sites at the 5' and 3' ends respectively (GenScript, USA).
Table 7.1. Primer sets used for mutagenesis of full length caveolin-1 gene

<table>
<thead>
<tr>
<th>Mutant name</th>
<th>Position of the mutation in CAV-A subdomain</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAV-1&lt;sup&gt;F92A-D82A&lt;/sup&gt;</td>
<td>Asp-82-Ala (D82A)</td>
<td>GGCCCTCCAGATGCC&lt;sup&gt;AGC&lt;/sup&gt; AAAAACTGTGTGTTCC</td>
<td>GGAACACACAGTGTGCT GCCATCTGGAAGGCC</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>CAV-1&lt;sup&gt;F92A-G83A&lt;/sup&gt;</td>
<td>Gly-83-Ala (G83A)</td>
<td>GCTGGCCTTCAGATGCC&lt;sup&gt;AGC&lt;/sup&gt; ATCAAAACTGTGTGT</td>
<td>ACACACAGTTTGATGCC ATCTGGAAGGCCAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAV-1&lt;sup&gt;F92A-I84A&lt;/sup&gt;</td>
<td>Ile-84-Ala (I84A)</td>
<td></td>
<td>Commercially Synthesized</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAV-1&lt;sup&gt;I84A&lt;/sup&gt;</td>
<td>Ile-84-Ala (I84A)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mutated bases are shown in bold.
7.3.3. Lentiviral plasmid construction and virus production

The sequence verified mutants were amplified by PCR introducing $BamH1$ and $Sal1$ sites at the 5' and 3' ends of the PCR product, respectively, via forward primer (ATCAGGATCCATGTCTGGGGGCA) and reverse primer (ATCAGTCTGACTTATATTTCCTTG) (restriction sites are underlined). The PCR product was then ligated into the pWPT-GFP lentiviral vector (Addgene) at the $BamH1$ and $Sal1$ restriction sites replacing GFP.

To construct lentiviral plasmids for I84A mutants, CAV-1$^{184A}$ and CAV-1$^{F92A-I84A}$ were double digested by $BamH1$ and $Sal1$, followed by cloning into the lentiviral plasmid pWPT at the $BamH1$ and $Sal1$ restriction sites and the successful cloning was confirmed by double digestion. The lentiviral vectors were then constructed as described elsewhere in this thesis (Section 2.4, Chapter 2).

7.3.4. DNA Sequencing

Mutant plasmids were sent to the Australian Genome Research Facility (AGRF, Sydney) and both sense and antisense strands of CAV-1 gene were sequenced using Sanger sequencing with CAV-1 specific sequencing primers (Sense strand: 5'-ATGTCTGGGGGCAATACGTA-3' and Antisense strand: 5'-GAAGGTGGTGAAGCTGCCCTT-3').
7.3.5. Bioinformatics

For the bioinformatics analysis, the open reading frame of the CAV-1 gene was opened using the CLC genomic workbench (Version 3.6 CLC Bio) and converted into corresponding amino acid sequence using ExPASy translate tool (http://web.expasy.org/translate/). To predict the effect of single mutations in the CAV-A subdomain on CSD hydrophobicity, amino acids at the positions, 82 (D), 83 (G) and 84 (I) were replaced with alanine and the mutated amino acid sequences were opened in CLC genomic workbench and hydrophobicity plots were generated using default parameters. Finally, the coiled-coils motif prediction was performed using COILED-COIL prediction tool (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_lupas.html) at ExPASy Bioinformatics resource portal.

To confirm the single amino acid mutation of the sequenced clones, the Sanger sequencing data sets were opened using CLC genomics workbench and then analyzed. The pairwise DNA and amino acid sequence alignments were performed using CLC genomic workbench and ClustalW at the EMBL-EBI bioinformatics resources (http://www.ebi.ac.uk/Tools/psa/).

7.3.6. Nitrite levels measurements

Nitrite levels were measured using the Griess reagent (Promega) according to the manufacturer’s instructions and measurement of absorbance at 540 nm. Triplicates of each sample were measured. Standard curve (from 0.1 µM to 100 µM) was
prepared with standard provided by the manufacture in the cell culture medium (DMEM).

7.3.7. Statistics

Data are presented as mean ± SEM. The statistical significance of differences was determined by one way analysis of variance (ANOVA) followed by post hoc. All tests were performed using the statistical software GraphPad Prism 6 (GraphPad, LaJolla, CA, USA). \( p < 0.05 \) was considered statistically significant.
7.4. Results

7.4.1. Substitution of isoleucine at the amino acid position 84 (I84) to alanine (I84A) can decrease CSD hydrophobicity

As previous data has demonstrated that the CAV-A subdomain (amino acids D82-S88) (Fig 7.1a) may have an effect of CSD solubility, the individual contribution of each of these six (except A87 in CAV-A) residues to hydrophobicity was predicted using an alanine scanning approach coupled with in silico analysis. Alanine scanning of residues and, replacement of I84 with alanine (CAV-1^{I84A}) was predicted to be lowest hydrophobicity in the CSD (CAV-1^{I84A}) (Fig 7.1b) among all of the alanine substituted mutants and wild type CAV-1.
Figure 7.1. Structure of CAV-1 scaffolding domain (CSD) and hydrophobicity of CAV-A subdomain. (a), Subdomains of CSD and their amino acid composition and localization. (b), Bioinformatics analysis of hydrophobicity of alanine substituted mutants of CAV-A subdomain. Red boxes indicate the levels of hydrophobicity of the CSD of wild type CAV-1.
7.4.2. DNA sequencing confirmed successful alanine substitution.

Since the CAV-1\(^{F92A}\) has been shown to remove CSD mediated eNOS inhibition, a double mutant was synthesized encoding the I84A and F92A mutations to test the effect on eNOS derived NO synthesis. As controls for this novel CAV-A mutant, two more amino acids in CAV-A subdomain (D82 and G83) were changed to alanine (D82A and G83A respectively) within the F92A CAV-1 backbone which resulted in CAV-1\(^{F92A-D82A}\) and CAV-1\(^{F92A-G83A}\) double mutant plasmids. Following mutagenesis, the size of the CAV-1 gene was confirmed by double restriction enzyme digestion yielding ~ 500 bp (Fig 7.2a and Fig 7.3a) fragments and successful mutations were confirmed by DNA sequencing of mutant plasmids. DNA sequencing confirmed alanine substitution at the amino acid positions 82 (Fig 7.2b) and 83 (Fig 7.3b) resulted in CAV-1\(^{F92A-D82A}\) and CAV-1\(^{F92A-G83A}\) double mutant plasmids.

Next, successful construction of lentiviral plasmids expressing mutated CAV-1\(^{F92A-D82A}\) (Fig 7.4a), CAV-1\(^{F92A-G83A}\) (Fig 7.4b), CAV-1\(^{F92A-I84A}\) (Fig 7.4c) and the single mutant CAV-1\(^{I84A}\) (Fig 7.4c) were also confirmed by double digestion of resulted plasmids yielding ~ 500 bp fragments.
Figure 7.2. Verification of (D82A) substitution. (a), following mutagenesis, the size of the mutated version of CAV-1 was determined by double digestion of the resulting plasmid, and (b), verification by DNA sequencing. Arrow points the successful point mutation in the nucleotide triplet encoding for alanine.
Figure 7.3. Verification of (G83A) substitution. (a), following mutagenesis, the size of the mutated version of CAV-1 was determined by double digestion of the resulting plasmid, and (b), verification by DNA sequencing. Arrow points the successful point mutation in the nucleotide triplet encoding for alanine.
Figure 7.4. Confirmation of cloning of CAV-1 mutant DNA fragments into the pWPT-GFP lentiviral plasmid. Mutant CAV-1 DNA fragments were ligated into a lentiviral vector, replacing the GFP sequence, of (a), CAV-1\textsuperscript{F92A-D82A}, (b), CAV-1\textsuperscript{F92A-G83A}, and (c), CAV-1\textsuperscript{F92A-I84A} and CAV-1\textsuperscript{I84A} ligation was confirmed by double digestion of the resulting plasmid.
7.4.3. I84A mutation in CAV-A subdomain does not enhance F92A mediated eNOS-driven NO synthesis

To investigate, the effect of CAV-A mutations on F92A mediated eNOS derived NO synthesis, HEK293T cells were transduced with lentiviral vectors expressing eNOS and a series of CAV-1 mutants, and 72 h post transduction, nitrite levels were measured by the Griess assay. In summary, no significant differences in nitrite levels were observed in HEK293TeNOS+CAV-1F92A-I84A cells compared to HEK293TeNOS+CAV-1F92A cells (Fig 7.5), suggesting that I84A mutation does not have any additive effect on F92A mediated eNOS derived NO synthesis. In addition, alternative CAV-1 double mutants (CAV-1\(^{F92A-D82A}\), and CAV-1\(^{F92A-G83A}\)) also did not show any significant difference compared to CAV-1\(^{F92A}\) in terms of eNOS derived NO release. On the other hand, nitrite levels in cells transduced with single mutant, CAV-1\(^{I84A}\) with eNOS (HEK293TeNOS+CAV-1\(^{I84A}\)) was not significantly different from wild type CAV-1\(^{WT}\) and eNOS transduced cells (HEK293TeNOS+CAV-1\(^{WT}\)) (Fig 7.5), suggesting that I84A mutation does not affect the CAV-1 interaction with eNOS.
Figure 7.5. Alanine substitution of amino acid residues of CAV-A subdomain revealed no enhancement of F92A mediated eNOS derived NO synthesis. Nitrite levels released from gene delivered and control cells were measured by Griess assay. *p<0.05 and #p<0.05 vs. eNOS+CAV-1I84A, eNOS+CAV-1WT, CAV-1F92A, CAV-1F92A-D82A, CAV-1F92A-G83A, CAV-1F92A-I84A, CAV-1F92A-G83A, CAV-1F92A-I84A-D82A, and un-transduced. N=3. One way ANOVA followed by post hoc
7.4.4. *In silico* analysis of alanine substituted amino acid residues in the CAV-A subdomain in terms of protein-protein interaction motifs

The impact of CAV-A mutations on protein-protein interaction motifs, Coiled-coil structures was predicted by bioinformatics analysis, in which mutated amino acid sequences were uploaded to COILED-COILS prediction tool and the probability of coiled-coil structure formation and relative positions were visualized using default parameters. Coiled-coil structure prediction of the four novel mutants with the CAV-A subdomain (CAV-1^{F92A-D82A}, CAV-1^{F92A-G83A}, CAV-1^{I84A}, and CAV-1^{F92A-I84A}) revealed that, the N-terminal coiled-coils motifs were exactly similar to CAV-1^{F92A} and wild type CAV-1 (CAV-1^{WT}) (Fig 7.6). This, *in silico* analysis suggests that the novel CAV-A double mutants may not have any impact on CAV-1 interaction properties.
Figure 7.6. *In silico* prediction of Coiled-coils motif of full length CAV-1. Coiled-coil motifs were predicted of full length CAV-1 mutants and CAV-1<sup>WT</sup> using the COILED-COIL prediction tool (ExPASy Bioinformatics Resource Portal). Green arrows indicate the predicted coiled-coils motifs and their locations in the CAV-1 protein.
7.5. Discussion

Genome scale studies have revealed that protein-protein interaction is an important phenomenon in cellular signal transduction pathways (Pawson and Nash, 2000, Tong et al. 2002), in which, ligand binding consensus sequences have been identified using phage display technology and generation of protein-protein interaction networks created computationally following *in silico* modeling. Yeast two hybrid systems have also been employed to confirm protein-protein interactions experimentally (Bair et al. 2008; Lancero et al. 2005) and SH3 domains have been identified as key interacting motifs in many signalling proteins such as Bem1, Sho1, and Boi1. On the other hand, methods to alter protein-protein interactions can provide a path to further understand the downstream effects of these protein functions within networks. Alanine scanning mutagenesis is a method for analyzing important interactions in protein-protein interfaces (Hayashi et al. 2015). Individual substitutions of interested amino acids with alanine allow deciphering of which interactions are critical in an interface and which ones are not (Clackson and Wells, 1995, Bernatchez et al. 2005, Hayashi et al. 2015).

CAV-1 has been shown to interact with other proteins such as PDGFRα/β (Yamamoto et al. 1999), EGFR (Couet et al. 1997), TGFβR1 (Razani et al. 2001), Integrin (Wei et al. 1999) and also with eNOS (Garcia-Cardena et al. 1997, Bernatchez et al. 2005; Trane et al. 2014) thus actively participating in signal transduction pathways such as PDGFR-Ras-ERK (extracellular signal-regulated kinase), Rac1 (Shao et al. 2013) and it has been proposed that CAV-1 may modulate these signal transduction pathways by attracting signalling molecules.
(Mineo et al. 1996). For instance, when EGF binds with the EGFR, Raf-1 is recruited to the caveolae and this recruitment can stimulate caveolae to release EGFR to regulate PDGFR-Ras-ERK signalling (Mineo et al. 1996). Furthermore, when CAV-1 interacts with eNOS, the CAV-1 scaffolding domain (CSD, D82-R101) has been shown to interact with $^{350}$FSAAPFSGW$^{358}$ peptide region of human eNOS (Couet et al. 1997; Garcia-Cardena et al. 1997), and further studies have revealed that there are three subdomains (CAV-A, CAV-B, CAV-C) within the CSD (Trane et al. 2014, Bernatchez et al., 2005). CAV-A and CAV-B subdomains have been shown to inhibit eNOS derived NO synthesis in cellular models. It has been shown that alanine substitution at position 92 within the CAV-B subdomain (CAV-1$^{F92A}$) can remove eNOS inhibition and increase NO production (Bernatchez et al. 2005). They have further reported that the CAV-A subdomain is non-inhibitory for eNOS whilst CAV-B has been shown to have an inhibitory effect. This indicated that peptides inhibitory to eNOS function are localized within the CAV-B subdomain and interestingly, co-delivery of full length CAV-A ($^{82}$DGIWKAS$^{88}$) and CAV-B ($^{89}$FTTFTVT$^{95}$) peptides showed greater inhibition compared to CAV-B alone and, it has been further shown that co-delivery of an F92A mutated CAV-B peptide ($^{89}$FTTATVT$^{95}$) together with full length CAV-A peptide ($^{82}$DGIWKAS$^{88}$) could significantly increase eNOS derived NO synthesis compared to F92A-CAV peptides alone (Bernatchez et al. 2005, Bernatchez et al. 2011), suggesting that the CAV-A subdomain may increase CAV-1$^{F92A}$ solubility and thus increase distribution and bioavailability to interact more efficiently with eNOS (Bernatchez et al. 2005). To understand which residues of CAV-A subdomain may play a role in
this phenomenon, an alanine scanning approach was performed, and
bioinformatics analysis revealed that an I84A mutation might decrease
hydrophobicity and therefore increase solubility of the CSD (Fig 7.1b).
Hydrophobicity can be increased with increasing number of carbon atoms within
the amino acid side chains (Nicolau et al. 2014). Comparing all of the amino acid
residues in the CAV-A subdomain showed that I84 has maximum number of C
atoms (6) whilst alanine has only 3 which may contribute to lower hydrophobicity
(Kumar et al. 2016). However, the experimentally generated double mutant
encoding F92A and I84A (CAV-1F92A-I84A) did not show any additive effect on F92A
mediated eNOS derived NO production, furthermore confirming this neutral effect
of I84A mutation, the single mutant encoding I84A (CAV-1I84A) also did not show
any significant difference compared to CAV-1WT in terms of eNOS mediated NO
release (Fig 7.5). This may be due to the possibility that the solubility contributed
by the non-mutated CAV-A subdomain is optimum and further modification does
not provide an additive effect on the F92A residue within the CAV-B subdomain
and efficient interaction with eNOS.

The coiled-coil motifs consist of two or more alpha helices twisted around one
another and are one of the most commonly found interacting motifs in nature
have demonstrated that mitochondrial division protein 1 (Mdv1) interacts with Fis 1
protein through a central coiled-coil motif and this Mdv1-Fis1 interaction has been
shown to be important for mitochondrial fission. On the other hand, coiled-coil
interactions can be reversible in some conditions such as temperature changes
(Rose and Meier, 2004), in which, at a temperature below 37°C, *Salmonella* coiled-coil protein TipA has been shown to form a dimer which can bind with DNA and act as a repressor and above 37°C, this coiled-coil dimer has been shown to unfolded resulting in release from the DNA and subsequent activation of respective gene transcription (Hurme et al. 1997). Many studies have shown that coiled-coil interactions are important for a variety of cellular functions such as assembly of protein complexes (Rose and Meier, 2004), transcription (O'Shea et al. 1991) and intracellular trafficking (Gillingham and Munro, 2003). Interestingly, Stoeber and colleagues have recently demonstrated that cavins interact with caveolins through coiled-coil motifs to form caveolae (Stoeber et al. 2016). On the other hand, coiled-coil motifs have been shown to be important for protein scaffolding function (Kuhn et al. 2014), and have also been shown to associate with membranes such as mitochondrial membranes (Zhang, et al. 2012). *In silico* analysis of the novel CAV-A mutants described in this study predicted that, alanine substitution in CAV-A subdomain does not have an impact on CAV-1 coiled-coil motifs (Fig 7.6), which may suggest that these mutations do not affect protein-protein interaction properties of CAV-1.

It has been previously shown that the CAV-B subdomain is composed of mainly hydrophobic residues (TTFTFTVT, Fig 7.1a) and CAV-B subdomain alone has been shown to be completely insoluble when eNOS activity was tested with purified recombinant eNOS protein and CAV peptides (Bernatchez et al. 2005), suggesting that CAV-B can strongly interact with hydrophobic residues
(\textsuperscript{350}FSAAPFSGW\textsuperscript{358}) of the eNOS oxygenase domain (Garcia-Cardena et al. 1997; Couet et al. 1997). Biochemical studies have further demonstrated that the CAV-1 CSD can interact with both the oxygenase and reductase domains of eNOS (Garcia-Cardena et al. 1997; Venema et al. 1997; Michel et al. 1997) and this interaction can inhibit the reductase function of the eNOS and reduce NO synthesis (Ghosh et al. 1998). More specifically, the F92 residue on the CAV-B subdomain has been shown to be involved in eNOS inhibition which may be due to the phenyl alanine residue containing an aromatic ring potentially disrupting electron flux since phenyl rings can accept and stabilize electrons through resonance structures (Bernatchez et al. 2005). Therefore when phenylalanine was substituted to alanine, the electron accepting aromatic rings disappear since alanine does not contain aromatic rings (Kumar et al. 2016), and thus the F92A mutation may increases electron flux and activation of eNOS.

7.6. Conclusions

The data from this study shows that single amino acid substitution to alanine residues in the CAV-A subdomain may not significantly impact on the solubility of CSD.
7.7. References


Figure 7.S1. CAV-1$^{F92A}$ mutant plasmid. A 534-bp fragment of the human caveolin-1 mutant in which phenyl alanine (F) at amino acid position 92 was replaced with alanine (A) and sub cloned into pMA-T backbone.
Chapter 8: General discussion
There is an increasing demand for cell therapy based approaches for the treatment of a large number of chronic diseases such as cardiovascular diseases and acute injuries including bone fractures and wounds (Gómez-Barrena et al. 2015; Hao et al. 2017; Puliafico et al. 2013), in which damaged tissues can be repaired through transplantation of functional stem cells either systemically or directly to the injured site. Despite the high capacity of ESC differentiation and their clinical potential, the use of ESCs has been questioned with ethical concerns associated with their isolation and safety concerns due to their potential to form teratomas (Jiang et al. 2014; Liu et al. 2013). In the landmark discovery of mouse iPS cells, Takahashi and Yamanaka (2006) developed an alternative cell source to mouse ESCs and showed that iPSCs resembled certain ESC characteristics giving rise to a full spectrum of cells types, and this finding was replicated in human cells with the generation of human iPSCs (Takahashi et al. 2007). However, the use of iPSCs is also associated with safety concerns such as teratoma formation after transplantation (Ben-David and Benvenisty, 2011; Liu et al. 2013). The use of mesenchymal stem cells (MSCs) is largely free of the issues associated with ESCs and iPSCs but their differentiation potential is limited compared to pluripotent stem cells and these cells may provide a more acceptable platform for cell therapy approaches given their potential for allogeneic transplantation and also their culture expansion ability. Genetic engineering provides a target cell with specific characteristics which may enhance possible therapeutic effects and provides a useful approach to improve stem cell based therapies. The first reprogramming study showed that ectopic expression of single muscle specific transcription factor,
MyoD in terminally differentiated fibroblasts allowed conversion of them into muscle cells showing that lineage specific genetic factors can be used to convert one cell type to another (Davis et al. 1987; Weintraub et al. 1989). Subsequently, others have shown that generation of clinically important cell types such as cardiomyocytes (Ieda et al. 2010), endothelial cells (Ginsberg et al. 2012; Han et al. 2014), β cells (Yang et al. 2011), and neurons (Treutlein et al. 2016) using ectopic expression of lineage restricted transcription factors together with small molecules suggest universal applicability. However, the efficiency of the current approaches remains low limiting their clinical application. To this end, this thesis focusses on the use of a signalling molecule, nitric oxide to promote differentiation and cell fate conversion.

8.1. Reconstitution of the eNOS-CAV-1 interaction through a non-inhibitory CAV-1 mutant increased cellular NO synthesis

Cellular concentration of NO has been shown to contribute to cellular differentiation or cell toxicity, in which higher concentration (0.5 mM) has been shown to be toxic for embryonic cardiomyocytes and lower concentrations (<50 µM) have been shown to promote cell proliferation and has also been shown to induce cardiomyocyte differentiation (Mujoo et al., 2008), and osteogenesis (Ehnes et al. 2015) highlighting that the cellular effect of NO is highly depend on concentration. Furthermore, low concentrations of NO (1–10 µM) have been shown to induce signalling pathways such as c-Src, IRS-1, phosphoinositide 3-kinase, and AKT
(Tejedo et al. 2004) and has also been shown to protect cells from apoptosis through activation of cell survival pathways, in which β-cells have been shown to be protected from apoptosis by inducing insulin-like growth factor-1 and insulin (Cahuana et al. 2008). Interestingly, in this current study, NO levels in eNOS gene modified cells were achieved within a low concentration range (<10 μM) and furthermore, relatively increased levels of NO which were produced with co-expression of eNOS and CAV-1F92A also were within this range. CAV-1WT is endogenously expressed and thus, some part of the overexpressed eNOS protein molecules can be associated with CAV-1WT and unbound eNOS may account for increased NO production. Supporting this hypothesis, it has been shown that increased NO levels occur when eNOS protein is released from inhibitory CAV-1WT, which was achieved by mutating the CAV-1-eNOS binding motif (350FSAAPFSGW358) by alanine substitution at amino acid residues F350, F355 and W358 (Garcia-Cardena et al. 1997). Co-expression of eNOS and CAV-1F92A could reproduce two active eNOS species within the cell which may account for increased NO synthesis, in which, one eNOS species could be unbound eNOS and other species could be non-inhibitory CAV-1F92A associated eNOS (eNOS-CAV-1F92A). On the other hand, CAV-1F92 may compete with CAV-1WT to associate with eNOS and this may account for decrease amounts of CAV-1WT associated inactive eNOS species (eNOS-CAV-1WT). This hypothesis is supported from previous findings, in which, both CAV-1WT and CAV-1F92A have been shown to have a similar binding capacity for eNOS as shown by crystal structure based molecular modeling, in which both CAV-1WT and CAV-1F92A show similar
orientation with regards to the eNOS active site and close proximity to the iron-binding heme domain showing similar binding properties with eNOS (Rosenfeld et al. 2002). Alternatively, it could be proposed that, CAV-1<sup>WT</sup> may act as an endogenous regulator of eNOS activity to maintain NO levels within non-toxic levels.

Recently, experiments using GST pulldown and co-immunoprecipitation data have shown that, eNOS binding motifs are located between CAV-BC sub domains (90-99 amino) (Trane et al. 2014) and CAV-A subdomain has been predicted to be important for solubility of the CSD (Bernbatchez et al. 2005). Moreover, F92A mutation in CSD has been shown to interact with eNOS while removing CSD mediated eNOS inhibition (Trane et al. 2014). Thus, it could be argued that, increasing the solubility of CAV-A through a mutation might further increase F92A mediated eNOS activation, and thus in this thesis, chapter 7 focused on examining such a mutation. Through <em>in silico</em> analysis of alanine substituted CAV-A mutants, an I84A mutation was predicted to be the lowest in terms of hydrophobicity and a double mutant was generated with I84A and F92A however, no significant effect on eNOS derived NO release was observed compared with the original F92A mutation. This phenomenon could be explained in such that, F92A mediated NO release is likely to be saturated in terms of the solubility provided by the CAV-A subdomain and further enhancement to this solubility might be redundant. CAV-1<sup>F92A</sup> mediated eNOS activation can be explained by removal of electron accepting resonance structures from the eNOS interacting interface of CSD through alanine substitution (Bernatchez et al. 2005), or the F92A mutation could mediate
decreased CAV-1 phosphorylation at the Tyr-14 amino acid residues. Chen et al (2012) have reported that increased Tyr-14 phosphorylation of CAV-1\textsuperscript{WT} promote eNOS inhibition due to strong binding with eNOS. Supporting this concept, Kimura et al (2002) have shown that over expression of a double mutant of CAV-1, in which substitution of both Phe-92 and Val-94 to alanine (F92A-V94A) can reduce Tyr-14 phosphorylation of both ectopically expressed CAV-1\textsuperscript{F92A-V94A} and endogenous CAV-1\textsuperscript{WT}.

8.2. Molecular control of NO synthesis enhances cellular differentiation

8.2.1. Osteogenesis

Chapter 3 describes the first evidence of simultaneous regulation of the β-catenin dependent canonical Wnt pathway and the non-canonical Wnt pathway via an eNOS-NO signalling pathway in adult mesenchymal stem cells to promote osteogenesis. The data agreed with previously shown reports where use of NO donors to promote osteogenesis has been demonstrated (Ding et al. 2012; Ehnes et al. 2015) in embryonic stem cells. Furthermore, eNOS knockout mice have been shown to have bone defects and errors in osteoblast maturation indicating the importance of NO in bone development (Armour et al. 2001; Aguirre et al. 2001). Furthermore, canonical Wnt signalling has been shown to promote osteogenesis, in which β-catenin nuclear localization upon canonical Wnt activation has been shown to induce bone development (Cai et al. 2016; Gaur et al. 2005). It has also been shown that β-catenin knockout mice have significant defects in bone.
formation (Ruiz et al. 2016). Interestingly, Wnt3a has been shown to induce osteoblast differentiation in MSCs (Si et al. 2006) whilst non-canonical Wnt5a has been shown to inhibit β-catenin (Mikels and Nusse, 2006) and, Hasegawa et al (2015) have demonstrated that Wnt5a could suppress osteogenesis of human periodontal ligament cells. It is also possible that NO may promote osteogenesis in association with induced angiogenesis, in which, inhibition of VEGF has been shown to reduce fracture healing in mice and conversely, exogenous VEGF can induce fracture healing via enhanced neovascularization (Street et al. 2002). Thus, it could be proposed that MSCs engineered to over-express eNOS may be a useful initial cell source to treat delayed healing in bone fractures.

8.2.2. Endothelial differentiation

Chapter 4 aimed to improve MSC endothelial differentiation by introducing eNOS and CAV-1F92A into MSCs via lentiviral vectors and the data suggests that increased levels of NO can convert rat MSCs into endothelial like cells in particular arterial like endothelial cells in normal growth medium without additional growth factors. eNOS is the isoform restricted to endothelial cells (ECs) and the expression of eNOS in ECs has been shown to be important for EC identity (Chan et al. 2004; Gan et al. 2005). In these studies, they have shown that eNOS gene expression in ECs is transcriptionally activated whilst in non-endothelial cells it is repressed due to histone deacetylation (Gan et al. 2005) and DNA methylation (Chan et al. 2004) and epigenetic silencing of eNOS is reversed by treatment with
the histone deacetylation inhibitor trichostatin A, and DNA methyltransferase inhibitor 5-azacytidine which resulted in induced endothelial commitment (Ohtani et al. 2011). Endothelial differentiation of rBMSC\textsuperscript{eNOS} or rBMSC\textsuperscript{eNOS+CAV-1F92A} cells was observed in normal growth medium and furthermore, NO has been shown to drive this process through Wnt/β-catenin signalling. Demonstrating the role of eNOS in vascular development, eNOS deficient mice have been shown to have significant vascular defects such as abnormal aortic valve developmental (Lee et al. 2000), defects in lung vascular development (Han and Stewart, 2006), impaired angiogenesis and wound healing (Lee et al. 1999). Wnt/β-catenin pathway has been shown to promote wound healing, angiogenesis and vascular development (Whyte et al. 2013; Liu et al. 2016a). For instance, using an ear wound mouse model, Whyte et al (2013) have demonstrated that, treatment with Wnt3a induced wound healing. The data from this thesis also demonstrated that, increased levels of NO may promote arterial specific endothelial cell generation through up regulation of Notch pathway transcription factors, such as Hey2. Inhibition of venous and lymphatic specific transcriptional programs were also was detected in this study and future work will investigate arterial EC marker expression such as ephrinB2 in vessels formed by transplanted cells \textit{in vivo}. In the \textit{in vivo} experimental approach to demonstrate neovessel formation, fibrin and polyurethane scaffolds were used and both have been previously shown to improve vessels formation \textit{in vivo} suggesting that they may act as a synergistic biomaterial for eNOS based cell therapy applications. Polyurethane scaffolds have been used in cardiac tissue engineering (Chiono et al. 2014) and bone tissue engineering (Bonzani et al. 2007)
to transplant respective cell types into the damaged areas and Polyurethane has been considered as a promising clinical grade biomaterial because of its biocompatibility, biodegradability, high flexibility, and excellent mechanical properties (Da Silva, 2010; Guelcher et al. 2008). Other candidate genes for reprogramming towards endothelial cells include, over expression of iPS factors (Oct3/4, Sox2, Klf4 and cMyc) in fibroblasts which leads to expression of VEGFR2 and KDR and subsequent incubation of these cells in endothelial differentiation medium results in ECs (Margariti et al (2012). A study by Han et al (2014) used 5 key factors (Foxo1, Er71, Klf2, Tal1, and Lmo2) to reprogram fibroblasts to ECs however the use of single gene to promote endothelial reprogramming as described in this study has not been previously published.

8.2.3. Cardiac reprogramming

The role of NO signalling in GMT driven cardiac reprogramming has not been previously investigated and in this study, establishment of an eNOS-CAV-1F92A interaction in human fibroblasts and eASCs showed enhanced GMT driven cardiac reprogramming and this appeared to be linked with endogenous induction of Wnt3a. In this study, NO mediated GMT driven cardiac reprogramming appeared to be directed towards atrial specific cardiomyocyte like cells as shown by gene expression data and generation of binuclear cardiomyocyte like cells. Atrial fibrillation (AF) has been shown to decline atrial mechanical function (Chugh et al. 2014) and current treatments are mainly antiarrhythmic drugs (Devalla et al. 2015).
However current antiarrhythmic drugs show a lack of atrial selectivity and may cause side effects such as ventricular proarrhythmia (Dobrev and Nattel, 2010) and developments of new drugs with enhanced atrial selectivity are needed. Candidate drug screening is restricted by dependence on primary atrial specific cardiomyocytes (Devalla et al. 2015). Another application of the findings of this study may be optimization of protocols using NO to generate atrial cardiomyocytes for use in *in vitro* drug screening models. It could also be proposed that, transplantation of reprogrammed fibroblasts which are committed to atrial like cardiomyocytes in damaged atrium could decrease the formation of fibrotic heart tissue to AF (Blank et al. 2009). Electrophysiological properties like spontaneous beating or calcium transients were not observed in the reprogrammed cells generated in this study. One of the possible explanations is that the differentiation medium could be improved to obtain fully functional cardiomyocytes by including growth factors such as VEGF-A, FGF2, BMP4 to the differentiation medium, only 25 µg/ml ascorbic acid was included to the growth medium used in this study. It should be noted that, the current study was designed as a proof of concept to demonstrate that NO may promote GMT mediated cardiac reprogramming, and therefore, very basic differentiation medium was used to see the real effect of NO on cardiac reprogramming. Previous cardiac reprograming studies have shown that growth factors like BMP4 can play a pivotal role in generating functional cardiomyocytes (Monzen et al. 1999; Kattman et al. 2011) and may also activate eNOS via increased phosphorylation at Ser-1179, (Gangopahyay et al. 2011). Furthermore, it has been shown that activation of Wnt signalling in the first phase
of differentiation (approximately first 4-7 days) and then addition of BMP4 increased generation of functional cardiomyocytes (Naito et al. 2006; Lian et al. 2012; Mehta et al. 2014). Additionally, a distinctive role for the canonical and non-canonical Wnt signalling pathways has been shown in cardiomyocyte differentiation of human ESCs, in which canonical Wnt3a and Wnt8a are important for mesoderm commitment and early induction of cardiomyocytes (day 3) and interestingly non-canonical Wnt5a was important for the second phase (after day 7) of differentiation and also for maturation of cardiomyocytes (Mazzotta et al. 2016) suggesting the stage specific role of canonical and non-canonical Wnt signalling. It has been shown that expression of eNOS is important for maturation of cardiomyocytes, in which eNOS deficient mice show delayed cardiomyocyte maturation (Lepic et al. 2006). Finally, it has been shown that cardiac differentiation can be markedly enhanced by providing a 3D environment for cells through use of extracellular matrix (Zhang et al. 2012), possibly, providing a microenvironment which may reflect the behavior of cells during normal development. Together, it could be proposed that, eNOS expression is important for cardiomyocyte differentiation and supplementation with Wnt5a and BMP4 at the later stages of differentiation and also providing an ECM substrate may increase differentiation efficiency and maturation of reprogrammed cardiomyocytes.
8.3. NO may affect DNA methylation and histone acetylation status

NO may act as an epigenetic regulatory molecule to drive cellular reprogramming events. Chromatin modifications such as DNA methylation (Liao et al. 2015) and histone acetylation (Piao et al. 2013) have been well documented as playing an important regulatory role in cell fate determination. With regards to DNA methylation in mammals, there are three DNA methyltransferase enzymes (Liao et al. 2015; Liu et al. 2016b), and this can lead to silencing of respective gene transcription (Liu et al. 2016b, Zhang et al. 2016). Therefore, modifying DNA methylation can influence cellular differentiation (Liu et al. 2016b) as has been shown through inhibition of DNMT1 to increase MSC differentiation towards endothelial cells (Zhang et al. 2016). In a separate study by targeted demethylation of the MyoD distal promoter using CRISPR/Cas9 editing in fibroblasts facilitated reprogramming towards muscle cells (Liu et al. 2016b). Interestingly, Tsai et al (2012) demonstrated that overexpression of Oct4 and Nanog can up regulate DNMT1 expression through direct binding with the DNMT1 promoter region which maintained an undifferentiated state in MSCs suggesting that expression of DNMT1 acts as a main epigenetic barrier for MSC differentiation. In chapter 4, results show that NO can inhibit DNMT1 but not DNMT3a and DNMT3b expression, which may highlight that NO can promote MSC differentiation through down regulation of DNMT1. Methylation patterns in developing embryos are mediated by two de novo methyltransferases, DNMT3a and DNMT3b (Okano et al. 1999) and interestingly maintaining these methylation patterns in adult cells can be mediated by DNMT1 (Jones and Liang 2009). More specifically, in embryonic
development, *de novo* methyltransferases DNMT3a and DNMT3b establish a CG methylation pattern in the genome and DNMT1 promotes copying of CG methylation from parental to daughter strand at replication forks (Cedarand and Bergman, 2009; Probst et al. 2009). Thus, it is possible that targeted down regulation of DNMT1 through eNOS derived NO may facilitate endothelial differentiation of MSCs, whether eNOS expression in MSCs drives targeted demethylation of endothelial specific promoters is an interesting scenario.

8.4. eNOS based cell therapy

The use of bone marrow derived endothelial progenitor cells (EPCs) manipulated to overexpress eNOS improves the function of pulmonary endothelium, and can prevent the progression of pulmonary arterial hypertension (PAH), in a rat model (Zhao et al. 2005). With the encouraging results obtained from these pre-clinical studies, a phase 1 clinical trial (NCT00469027) (Granton et al. 2015) using autologous EPCs expressing eNOS has been performed and the results have demonstrated a trend in improvement of total pulmonary resistance during the EPC delivery period (3-day). Following this phase 1 clinical trial, a phase 2 clinical trials (NCT03001414) has been initiated to treat 45 patients with PAH using autologous EPCs expressing eNOS, with trial completion in 2021. MSCs are attractive for cell-based therapy and engineering of MSCs with eNOS may provide a therapeutic source of nitric oxide. In this context, minicircle (MC) based non-viral systems to deliver eNOS are emerging as a safe gene transfer vector (Chang et al. 2014) due
to the lack of bacterial backbone sequences e.g. antibiotic resistance genes, origin of replication and other inflammatory sequences (Hou et al. 2016) which may contribute to sustained transgene expression due to lower activation of nuclear transgene silencing mechanisms (Chen et al. 2003). Munye and colleagues (2016) have recently demonstrated that delivery of equimolar amounts of DNA to mouse lung using a MC vector could reduce inflammatory response and provided prolonged transgene expression as evident by luciferase activity for 2 weeks compared to conventional plasmids. This could be explained by removal of CpG bacterial motifs from the MC backbone, in which, CpG motifs have been shown to associate with histone proteins and this may lead to heterochromatin conformation and thus delivered transgene can be silenced due to inaccessible transcription factors when this phenomenon is occurring at the promoter of the transgene (Riu et al. 2007). The data resulting from this thesis (Chapter 6) shows enhanced gene delivery efficiency (both GFP and eNOS) to rBMSCs for an MC vector compared to a parental plasmid and furthermore, highlighting its therapeutic effects, MC-eNOS delivered cells exhibited enhanced angiogenic responses and increased endothelial commitment which may clearly suggest the importance of MC vector for accelerating the efficiency of future eNOS-cell therapy applications. To this end, it would be important to obtain more prolonged eNOS expression in the cells delivered to patients to enhance therapeutic effects and furthermore, it could be proposed that, MC based co-transfection of CAV-1F92A mutant with eNOS would be a novel approach since co-expression of CAV-1F92A has been shown to enhance eNOS activation. Supporting this concept, in a recent report has shown that,
pulmonary arterial pressure was significantly reduced in PAH rats treated with eNOS and CAV-1$^{F92A}$ delivered rBMSCs compared to eNOS alone (Chen et al. 2016), and they further showed increased NO levels in the systemic circulation of the rBMSCs$^{eNOS+CAV-1F92A}$ treated rats.

8.5. Future directions

8.5.1. Genome editing and cell therapy

Exogenous therapeutic transgenes can be delivered to cells through a variety of methods and an alternative approach is emerging, in which endogenous gene activation at their native genomic loci is possible.

The transactivation domain of Herpes simplex virus protein 16 (VP16) (Baron et al. 1997), and its multiple repeats (VP64) has been employed to target promoters or enhancers of endogenous genes to activate them together with, an engineered version of clustered, regularly interspaced, short palindromic repeat (CRISPR) system of *Streptococcus pyogenes* (Jinek et al. 2012). This has been performed for genes such as Col1A1 in mouse embryonic stem cells (Cheng et al. 2013). In this type II CRISPR system, the Cas9 protein is directed to desired genomic loci by short guide RNAs, where Cas9 can be activated as an endonuclease. In the engineered CRISPR/Cas9 system, the naturally found two noncoding CRISPR RNAs (crRNAs), including a trans-activating crRNA (tracrRNA) and a precursor crRNA (pre-crRNA) (Jinek et al. 2012) have been fused to a single guide RNA (gRNA) which can direct Cas9 to its target genomic site. In addition, in order to use
CRISPR/Cas9 system in gene activation, the endonuclease activity of Cas9 has been deleted by making double mutant Cas9 (D10A and H840A) and termed as dead Cas9 (dCas9) (Jinek et al. 2012; Gilbert et al. 2013). Using this system together with the VP64 transactivation domain, endogenous activation of MyoD in mouse embryonic fibroblasts has been shown to promote conversion of fibroblasts to skeletal myocytes (Chakraborty et al. 2014), and the system has also been employed to activate endogenous VEGF-A in human HEK293 cells with dCas9-VP64 and a single gRNA (Maeder et al. 2013).

Interestingly, when dCas9 was fused to transcriptional repressor domain KRAB (Kruppel associated box domain of Kox1) it has been shown to repress endogenous genes such as CD71 and CXCR4 in HeLa cells using single gRNAs specific to each of the gene promoters (Gilbert et al. 2013). In the context of direct lineage conversion, activation of neuronal lineage specific endogenous Brn2, Ascl1, and Myt1 transcription factors (BAM factors) using both N-terminal and C-terminal VP64 fused dCas9 system (VP64-dCas9-VP64) together with individual BAM targeted guide RNAs could convert mouse embryonic fibroblasts to induced neuronal cells (Black et al. 2016). Together it could be proposed that multiplex activation of cardiac reprogramming factors (GMT) together with eNOS might be a new approach for generation of cardiomyocytes and also, single gene activation of eNOS in MSCs would be better platform for cell therapy for the treatment of chronic diseases. Finally, efficient and safe vector systems to deliver the CRISPR/dCas9 components to target cells are needed and, lentiviral vectors are efficient systems for transduction and as an example, the CXCR4 receptor in
human CD4+ cells has been disrupted by the CRISPR/Cas9 system to protect human cells from Human Immunodeficiency Virus infection (Hou et al. 2015). Integrase-deficient lentiviral vectors are a potentially safer approach to transiently deliver Cas9 to target cells, which may allow reduced nuclease activity in cells (Lombardo et al. 2007; Genovese et al. 2014). Furthermore, lentiviral vectors also exhibit sufficient capacity to package nucleases and multiple gRNA expression cassettes for multiplex editing at several loci, in which simultaneous activation of IL1RN and HBG1 genes in HEK293T cells using a dCas9-VP64 together with respective gRNAs has been reported (Kabadi et al. 2014). With regards to the in vivo gene delivery, AAV is a superior gene transfer system in terms of safety, however, its packaging capacity is limited (less than ~4.8 kb of DNA) which may limit AAV application in CRISPR mediated genome editing since the commonly used S. pyogenes Cas9 is ~4.2 kb (Cong et al. 2013). The recent development of a smaller Cas9 system (~3.1 kb Cas9 from S. aureus) has been shown to be compatible with AAV packaging limits and has been used in the development of CRISPR-Cas9-based gene therapy (Ran et al. 2015; Friedland et al. 2015), in which a single AAV vector was capable of editing the cholesterol regulatory gene PcsK9 in mouse liver.

8.6. Concluding remarks

The results from this thesis provide an insight into the novel role of nitric oxide in cellular reprogramming events which can be achieved through maintaining NO
concentration at low levels (≥15 μM). When the optimum NO levels were maintained in a cellular environment through genetic engineering of the eNOS-NO pathway together with a non-inhibitory partner CAV-1<sup>F92A</sup>, endogenous Wnt signalling appears to play a role in regulation of stem cell differentiation towards osteoblasts, endothelial-like and cardiomyocyte-like cells. Furthermore, it has been observed that these optimum levels of NO synthesis in stem cells may modify the epigenomic landscape in which DNA methylation and histone deacetylation patterns govern cell fate. Further studies are warranted to fully investigate these epigenetic mechanisms which may highlight a novel NO mediated mechanism of cellular differentiation and may allow more precise manipulation towards specific cell lineages.
8.7. References


80. Si W, Kang Q, Luu HH, Park JK et al. CCN1/Cyr61 is regulated by the canonical Wnt signal and plays an important role in Wnt3A-induced osteoblast differentiation of mesenchymal stem cells. Mol Cell Biol. 2006;26(8):2955-64
Appendices
## Appendix 1

List of clinical trials has been performed with MSCs for cardiovascular disease.

<table>
<thead>
<tr>
<th>Clinical Trial ID</th>
<th>Disease</th>
<th>Phase</th>
<th>No. of patients/ Status</th>
<th>MSC source</th>
<th>Country</th>
<th>Conclusion</th>
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<td>Status</td>
<td>Source</td>
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<td>Greece</td>
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</tr>
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<td>Greece</td>
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<td></td>
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<td>South Korea</td>
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<td></td>
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<td>Allogenic</td>
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<td>45 / Recruiting</td>
<td>Allogenic</td>
<td>United States</td>
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<tr>
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<td>Title</td>
<td>Phase</td>
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<tr>
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<tr>
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Appendix 2

Sequence information for 343-bp long Runx2 enhancer conserved within mouse, human, dog, horse, opossum, and chicken and Hsp68 minimal promoter fused construct

5' TTATCATGTAATTATAATGATGATCATTACAAATCAGATGTTTATCTGGTTTTGAGTTTAAAGAG CTAAGCAGAGTTTTTAAAATCAACACATATGTGTCTTTTTCTGTATTTGGAAAGAAACAT TCTGCAATAATGCACAAAAGCGACCATAATTCTACAGTCATAATAACACCTGTAATTGACTTT TGGGGGTTGTTTTACTCTATATCTATTTTTGACCAGTAGAAACAGCAATGATGTGTGAAAA GCCCAAAATGCAAGTGCGCTTTCGCTGGGCTGCTGGGACAGACCTCTAGTTCCTAAATTAGTCCATGAGGTGTCAG AGGCAGCAGCTCCATTGTAACCCGATTGGGAGGATACGTACCCGACAGCGCCCCAGGCA TCTTCTCGGTTCTTAACTTTGGGCGGGAAGAATTTTAAATGCGTTTAAACCC CATATTACGAAGCTCAGTGCAGTTGTCGAAGAAGGGCCACAAAATCAACAACAACTGACACAGCAAC AGCTAGAGGTAGTCTTTTCTGCTCAGTTCACACAGCGCTTGAATTGCTCTAGCAGGCATAGCAGC AGTGTCACTAGTAGCACAGCAGCCTTCCACACACCTTCCCCCTTCGAGATCCGATCTTCCAGT GAACCCCGACAAACTCTTCTGGAAGATTCTATGCAAAAGGCGGAAACCAACAATCCCGAGTTTCAAA GGAGGCGGCGGGAGTCCACACAGACGCAAGACTGCTCTGAGTAGATTTGGCCCAAGCCCCGCTT CTCTGGCTCTGATTTGCGCAAGAGGTGGGGGCTGGGCTGGGCGGGTGAGAAGACTCCTTAAAGGCG GCAGGCGGCCAGCGAGGTGACAGCTCAGAGCGCTTCATCTCAGACAACAAATCTGGTTTCCGCC AGACACAGCAAGACAAGAGAACAGACGAGCGACGCGCCTTCGATCTGGCCAGGAC CAGGGGCTCCAGAGAGCCTCCTGGGCGGAGCGCAGCAGATCTCCTCGGAGCCGACG GAGCGCAGCAGGAGCATCCACGCGCGCGCGGAGCGCAGCGCTTCCGAGAAGCAGTAGCAGCGCG GCGCCATGGC 3'

Green letters represent 343-bp long Runx2 enhancer and black letters represent Hsp68 minimal promoter
Molecular control of nitric oxide synthesis through eNOS and caveolin-1 interaction regulates osteogenic differentiation of adipose-derived stem cells by modulation of Wnt/β-catenin signaling

Nadeeka Bandara1,2, Saliya Gurusinha1,5, Shiang Yong Lim2,3, Haying Chen4, Shuangfeng Chen4, Dawei Wang4, Bryan Hilbert5, Le-Xin Wang14 and Padraig Strappe1

Abstract

Background: Nitric oxide (NO) plays a role in a number of physiological processes including stem cell differentiation and osteogenesis. Endothelial nitric oxide synthase (eNOS), one of three NO-producing enzymes, is located in a close conformation with the caveolin-1 (CAV-1WT) membrane protein which is inhibitory to NO production. Modification of this interaction through mutation of the caveolin scaffold domain can increase NO release. In this study, we genetically modified equine adipose-derived stem cells (eASCs) with eNOS, CAV-1WT, and a CAV-1PDA (CAV-1WT mutant) and assessed NO-mediated osteogenic differentiation and the relationship with the Wnt signaling pathway.

Methods: NO production was enhanced by lentiviral vector co-delivery of eNOS and CAV-1PDA to eASCs, and osteogenesis and Wnt signaling was assessed by gene expression analysis and activity of a novel Runx2-GFP reporter. Cells were also exposed to a NO donor (NDonate) and the eNOS inhibitor, L-NAME.

Results: NO production as measured by nitrite was significantly increased in eNOS and CAV-1PDA transduced eASCs (+559 ± 22 μM) compared to eNOS alone (481 ± 99 μM) and un-transduced control cells (0.91 ± 0.23 μM, p < 0.05). During osteogenic differentiation, higher NO correlated with increased calcium deposition, Runx2, and alkaline phosphatase (ALP) gene expression and the activity of a Runx2-GFP reporter. Co-expression of eNOS and CAV-1WT transgenes resulted in lower NO production. Canonical Wnt signaling pathway-associated Wnt3a and Wnt8a gene expressions were increased in eNOS-CAV1PDA cells undergoing osteogenesis whilst non-canonical Wnt5a was decreased and similar results were seen with NONOate treatment. Treatment of osteogenic cultures with 2 mM L-NAME resulted in reduced Runx2, ALP, and Wnt3a expressions, whilst Wnt8a expression was increased in eNOS-delivered cells. Co-transduction of eASCs with a Wnt pathway responsive lentivirus reporter only showed activity in osteogenic co-transfected with a doxycycline inducible eNOS. Lentiviral vector expression of canonical Wnt3a and non-canonical Wnt5a in eASCs was associated with induced and suppressed osteogenic differentiation, respectively, whilst treatment of eNOS-osteogenic cells with the Wnt inhibitor Dkk-1 significantly reduced expressions of Runx2 and ALP.

Conclusions: This study identifies NO as a regulator of canonical Wnt/β-catenin signaling to promote osteogenesis in eASCs which may contribute to novel bone regeneration strategies.
Background
Mesenchymal stem cells (MSCs) have been isolated from various tissues such as adipose [1], heart [2], bone marrow [3, 4], and blood [5–9], and have the potential to differentiate into different lineages, including osteoblasts, chondrocytes, and adipocytes [10, 11]. The osteoblast differentiation program of MSCs is switched on by cell recruitment, and timely expression of genes including Runx2, alkaline phosphatase (ALP), type I collagen (ColAI), and osteocalcin (OC) followed by extracellular matrix mineralization [12]. This process can be induced by soluble molecules such as bone morphogenetic proteins (BMPs) [13] or Wnts [14–16] that activate several pathways and various downstream signals such as protein kinase [17] and growth factors [18] to trigger osteoblast differentiation of mesenchymal stem cells.

Nitric oxide (NO) is a signaling molecule with a short half-life [19, 20]. It can react within the cell where it is produced or penetrate cell membranes to affect adjacent cells [21]. NO exerts a variety of physiological effects such as regulating blood pressure via smooth muscle relaxation [22], mediating immune responses [23], controlling cell proliferation [24], modulating apoptosis [20], promoting growth factor-induced angiogenesis [4, 21], accelerating wound healing [4, 25] and functioning as a neurotransmitter [26]. These reported effects are mediated through activating the primary NO effector soluble guanylyl cyclase to produce cGMP [27] by NO-based chemical modifications of proteins through S-nitrosylation [28] or through epigenetic modification [29]. NO is known to play an important role in bone homeostasis. It is generated by many cell types present in the bone environment, most notably the osteoblast [30].

NO is synthesized from l-arginine by three isozymes of nitric oxide synthase (NOS), including neuronal NOS (nNOS), endothelial NOS (eNOS), and cytokine-inducible NOS (iNOS) [31]. Both iNOS [32, 33] and eNOS [34] have been shown to play a role in osteoblast differentiation. Mice lacking eNOS have shown marked bone abnormalities due to impaired osteoblast differentiation resulting in poor maintenance of bone mass [35, 36]. Gene expression data from neonatal calvarial osteoblasts from eNOS−/− mice have shown downregulation of Runx2, Csf-1, and osteocalcin [37]. On the other hand, high concentrations of NO released due to the pathological iNOS expression promote bone resorption through induced osteoclastogenesis [38]. Therefore, an optimum level of NO is important to drive osteogenic differentiation of the MSCs.

In contrast with other NOS family members, eNOS is localized mainly in specific intracellular domains, including the Golgi apparatus [39] and plasma membrane caveolae [40, 41]. A previously demonstrated direct interaction of eNOS with wild-type caveolin-1 (CAV-1WT) [42] has proposed that CAV-1WT functions as an endogenous negative regulator of eNOS [43]. In this context, eNOS binds to the caveolin-1 scaffolding domain (CSD; amino acids 82–101) [44] and, furthermore, Thr-90 and Thr-91 (T90 and T91), and Phe-92 (F92) were identified as critical residues for eNOS binding and inhibition [41]. Genetic modification of endothelial cells through overexpression of a mutated version of CAV-1 with a phenylalanine to alanine substitution at the amino acid position 92 (CAV-1F92A) resulted in increased NO production, overcoming the inhibitory effect of CAV-1WT [41].

In the present study, we tested the hypothesis that molecular control of NO synthesis in equine adipose-derived stem cells (eASCs), can promote osteogenic differentiation where endogenous eNOS is not available, by recreating the interaction between eNOS and CAV-1 (CAV-1WT and CAV-1F92A) regulates the osteogenic differentiation of eASCs. Our results indicate that the optimum level of NO induces osteogenic differentiation through activation of the downstream canonical Wnt/β-catenin signaling pathway.

Methods
Cell culture
eASCs were isolated from subcutaneous adipose tissue as previously described [45]. All sampling was carried out using protocols approved by the Charles Sturt University Animal Care and Ethics Committee. Human embryonic kidney 293 T cells (HEK293T) (ATCC, VA, USA) and eASCs were cultured and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Bovogen, VIC, Australia), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine (Invitrogen) (growth medium) at 37 °C and 5% CO2.

Plasmid constructs
The list of cDNA for the genes of interest used in this study is listed in Table 1, and was used for construction of lentiviral vectors. HF Phusion (New England Biolabs; NEB) DNA polymerase was used for all the polymerase chain

Table 1: Lentiviral vectors and reporter constructs used in this study

<table>
<thead>
<tr>
<th>Lentiviral vector</th>
<th>Relevant properties</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWPT-eNOS</td>
<td>CMV-eNOS</td>
<td>This study</td>
</tr>
<tr>
<td>pWPT-CAV-1&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>CMV-CAV-1&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>This study and [81]</td>
</tr>
<tr>
<td>plXX-CAV-1&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>CMV-CAV-1&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pTRP-Runx2-Hsp68-eGFP</td>
<td>Runx2-Hsp68-eGFP</td>
<td>This study and [86]</td>
</tr>
<tr>
<td>pJX04-Runx2-Hsp68-eGFP</td>
<td>Runx2-Hsp68-eGFP</td>
<td>This study</td>
</tr>
<tr>
<td>pJX04-Runx2-Hsp68-eGFP</td>
<td>Runx2-Hsp68-eGFP</td>
<td>This study</td>
</tr>
<tr>
<td>pJX04-Runx2-Hsp68-eGFP</td>
<td>Runx2-Hsp68-eGFP</td>
<td>This study</td>
</tr>
<tr>
<td>pSL-TGF/LEF-eGFP</td>
<td>TGF/LEF-eGFP</td>
<td>Addgene (#14745)</td>
</tr>
</tbody>
</table>

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reactions (PCRs) and all the restriction endonucleases were purchased from NEB unless indicated otherwise.

To construct the CMV promoter-driven eNOS expressing lentiviral vector, a codon-optimized eNOS gene was synthesized [4] and subcloned into the pWPT-GFP lentiviral plasmid (Addgene, MA, USA) using BamHI and SalI restriction endonucleases. Doxycycline (DOX) inducible eNOS construct was prepared by amplifying the eNOS gene using the forward (ATCGAGATTCATGGGCAACCTGAA) and reverse primer (ATCGAGGTGTGACATGGGCTG) by introducing EcoRI restriction sites (underlined in both forward and reverse primers) at both the 5' and 3' ends of the final PCR product, followed by subcloning the PCR product into FUW-TetO vector (Addgene). Human wild-type caveolin-1 (CAV-1WT) expressing lentiviral vector was constructed by inserting the full length CAV-1WT (Addgene) into pLVX-A6GFP-C1 and pLVX-DsRed-C1 (Clontech, CA, USA) using EcoRI and BamHI restriction endonucleases. A mutated caveolin-1 (CAV-1mut) in which phenylalanine (F) at the amino add position 92 was replaced with alanine (A) [41] was synthesized (GenScript), amplified by PCR by introducing BamHI and SalI sites at the 5' and 3' ends of the PCR product, respectively, via forward primer (ATCGAGCTACATGGGCTG) and reverse primer (ATCGAGTACATGGGCTG) (restriction sites are underlined). The PCR product was then ligated into the pWPT-GFP lentiviral vector (Addgene) at the BamHI and SalI restriction sites replacing GFP. Wnt3a and Wnt5a expressing lentiviral plasmids were purchased from DNAuo plasmid repository.

**GFP reporter constructs**

A 343bp fragment of the Runx2 enhancer region (sequence information was kindly provided by Toshihisa Komori at the Department of Cell Biology, Nagasaki University) [46] was synthesized together with the sequence of the Hsp68 minimal promoter (GenScript, NJ, USA). The entire fragment was then subcloned into pTrc99A-GFP lentiviral vector at the MluI and BamHI restriction sites replacing an insulin-specific promoter, upstream of the enhanced GFP (eGFP) coding sequence. The Wnt responsive lentiviral TCF/LEF-dGFP reporter system was purchased from Addgene.

**Lentiviral vector production and transduction of equine adipose stem cells**

Lentiviral vectors used in this study (Table 1) were generated by four plasmid transfection of HEK293T cells. Briefly, each well of a six-well tissue culture plate was coated with 50 μg/mL of dI-hyaluronic acid (Sigma-Aldrich) in phosphate-buffered saline (PBS) and incubated for 2 h at 37 °C. HEK293T cells were then seeded at a density of 1 x 10⁶ cells per well, 24 h prior to transfection of 6.3 μg of packaging plasmid pSPAX2 (Addgene), 3.1 μg of Rev expression plasmid pRSV Rev (Addgene), 3.5 μg of VSV-G envelop pMD2.G (Addgene), and 10 μg of the gene of interest expression transfer vector using a standard calcium phosphate transfection method [47]. Seventeen hours post-transfection, the media was changed and supernatant containing lentiviral vectors were collected at 48 h and 72 h post-transfection, combined, and filtered through a 0.45-kμM PVDF filter, and used for eASC transduction in the presence of 4 μg/mL Polybrene (Sigma-Aldrich).

**Osteogenic differentiation**

eASCs were seeded in a 12-well plate (11,000 cells/cm²) in triplicate in growth medium overnight followed by transduction with eNOS, CAV-1WT, and CAV-1mut lentiviruses. After 3 days, growth medium was replaced with osteogenic induction medium (OM; growth medium + 0.2 mM 2-phospho-l-ascorbic acid trisodium salt + 10 nM dexamethasone + 10 mM β-glycerol phosphate; Sigma-Aldrich). Non-induced control cells were cultured in growth medium. Medium was changed every 3 days (see Fig. 2a below).

After 11 days incubation in OM or control growth medium, cells were washed with PBS and fixed with 4% (v/v) paraformaldehyde (Sigma-Aldrich) for 20 min, washed with distilled water, and then stained with 2% (w/v) Alizarin Red S (pH 4.2) for 20 min. Stained cells were washed with distilled water prior to assessment by light microscopy using a Nikon Eclipse Ti-S inverted microscope (Nikon, Japan).

**Alizarin Red S quantification**

Quantification of Alizarin Red S staining was performed as previously described [48]. Briefly, after staining the cells with Alizarin Red S for 20 min, 10% acetic acid was added to the 12-well cell culture plate and incubated for 30 min with shaking. The Alizarin Red S stain was extracted and the absorbance was measured at 405 nm in parallel with Alizarin Red S standards comprising of serial 1:2 dilutions of 50 mM Alizarin Red S (pH 4.2).

**Quantitative real-time PCR**

Total RNA from transduced and control cells after 11 days of incubation in OM or growth medium was isolated using the PureZol reagent (Bio-Rad, CA, USA) according to the manufacturer's instructions, and the concentration of isolated RNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific), treated with RNase-free DNase (1 U/1 μg RNA; Promega, WI, USA). cDNA was synthesized with 1 μg RNA from all samples using a High Capacity Reverse Trancription Kit (Thermo Fisher Scientific). Quantitative real-time PCR assays were performed on a BioRad CFX96 Real-Time system (Bio-Rad) using the ScFast EvaGreen Supermix (Bio-Rad). Primer sequences used for target gene amplification are described in Table 2. Assays were performed in triplicate and target
Table 2: Primers used for reverse transcription quantitative polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>ATGGGTATGTTAATTCCTGCTC</td>
<td>AGCTTTGCAACTGATATGAC</td>
<td>NM_001081838</td>
</tr>
<tr>
<td>Runx2</td>
<td>TCCACACGCGCAGTGTCT</td>
<td>TAAGTGGATGAGGATAAATGCT</td>
<td>XM_005603686</td>
</tr>
<tr>
<td>ALP</td>
<td>TACGGAACATCTCGAACAGGAG</td>
<td>GTCTAAGGCGAGCCACGAG</td>
<td>XM_008537963</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>TCAAGATCAGCCTGAGGAG</td>
<td>GCTTCCTTGATGACGAG</td>
<td>XM_014730421</td>
</tr>
<tr>
<td>Wnt5a</td>
<td>CCCAGGCGTAGTCTGCTGTAGCCAA</td>
<td>AGCTCTGTGGTGAGAG</td>
<td>XM_014730656</td>
</tr>
<tr>
<td>GFAP</td>
<td>ACGTCTCGACGCCATGACAG</td>
<td>TATCAGTATGATCGACGAA</td>
<td>XM_014731495</td>
</tr>
</tbody>
</table>

Gene expression was normalized to equine β-actin mRNA levels using the ΔΔCt method.

Immunocytochemistry and confocal microscopy

Immunocytochemical detection of eNOS and caveolin-1 (Cav-1 WT and Cav-1 PRA) expression in eASCs was performed as follows. Briefly, cells were fixed in 4% paraformaldehyde for 20 min at 37 °C, treated with 0.1% Triton-X100 in PBS for 10 min, and blocked in a 10% PBS solution for 30 min at room temperature. This was followed by a 2-h incubation with a primary mouse monoclonal anti-eNOS antibody (BD Biosciences, CA, USA) or rabbit polyclonal anti-Cav-1 antibody (Cell Signaling Technology, MA, USA), and subsequently with an anti-mouse IgG secondary antibody conjugated with Alexa 488 (Cell Signaling Technology) or an anti-rabbit IgG secondary antibody conjugated with Alexa 555 (Cell Signaling Technology) for 1 h and counterstained with DAPI for nuclear staining (Sigma-Aldrich). eNOS and Cav-1 co-localization was observed by confocal microscopy (Nikon).

To detect β-catenin expression, eNOS transfected cells (with or without DOX treatment) and un-transfected cells were fixed in 4% paraformaldehyde for 20 min at 37 °C, treated with 0.1% Triton-X100 in PBS for 10 min, and blocked in a 10% PBS solution for 30 min at room temperature. This was followed by an overnight incubation with a primary rabbit monoclonal anti-β-catenin antibody (Cell Signaling Technology) and subsequently with an anti-rabbit IgG secondary antibody conjugated with Alexa 488 (Cell Signaling Technology) for 1 h and counterstained with DAPI.

**GFP reporter assays**

For GFP-based reporter assays for both TCF:LEF-dGFP and Runx2:Hsp68-eGFP, cells transfected with the TCF/LEF-dGFP and Runx2:Hsp68-eGFP were subjected for reverse transcription quantitative PCR (RT-qPCR) for GFP expression and fluorescence microscopic analysis, respectively.

**Nitric oxide detection**

Extracellular NO production was measured using the Griess reagent (Promega) according to the manufacturer's instructions and measurement of absorbance at 540 nm. Triplicates of each sample were measured at each time-point during osteogenic differentiation from day 0 to day 11.

**Statistical analysis**

All experiments were performed in triplicate and at least three times. Data were presented as mean ± SEM. The statistical significances were determined by one-way analysis of variance (ANOVA) followed by Tukey's test. All tests were performed using the statistical software GraphPad Prism 6 (GraphPad, CA, USA). p < 0.05 was considered statistically significant.

**Results**

**eASC characterization**

eASCs were spindle-shaped and adherent to plastic tissue culture dishes (data not shown). We have previously reported their tri-lineage differentiation potentials [49].

**eNOS and caveolin-1 expression in eASCs**

eNOS activation is controlled at the cell plasma membrane significantly by Cav-1, a major protein in caveola [50, 51]. We investigated eNOS and Cav-1 expression in un-transfected and transduced eASCs by immunofluorescence microscopy. Wild-type Cav-1 (Cav-1 WT)-transduced eASCs (eASC(Cav-1 WT)) and un-transfected eASCs (eASC(WT)) expressed Cav-1 protein; notably, the Cav-1 expression was increased in eASC(Cav-1 WT) (Fig. 1d) compared to eASC(WT) (Fig. 1c). Interestingly, eNOS expression was absent in eASC(WT) (Fig. 1a), whereas strong eNOS expression was observed in eNOS-transfected cells (eASC-NOx) (Fig. 1b). Next, we examined the localization of eNOS, Cav-1 WT, and mutated Cav-1 (Cav-1 PRA) in genetically modified eASC by confocal microscopy. As expected, eNOS expression was detected at the cytoplasm (Fig. 1e), whereas both the Cav-1 WT (Fig. 1f) and Cav-1 PRA (Fig. 1g) expressions were observed at the plasma membrane, confirming that F92A mutation of Cav-1 does not affect its cellular localization. Co-localization of eNOS and Cav-1 PRA was examined in co-transfected eASCs with eNOS and Cav-1 PRA (eASC-eNOS+cav-1 PRA) by confocal microscopy with expression of eNOS in the cytoplasm and Cav-1 PRA at the plasma membrane (Fig. 1h). As controls for primary antibodies, immunostaining was carried out in
the absence of primary antibodies specific to eNOS and CAV-1 (Additional file 1: Figure S1).

**NO enhances osteogenic differentiation**

To examine the role of the NO signaling in eASCs osteogenesis, we first compared the osteogenic differentiation between eNOS transduced (eASC\textsuperscript{NOS}) and untransduced eASCs (eASC\textsuperscript{WT}). A greater number of Alizarin Red S-positive nodules were induced in the eASC\textsuperscript{NOS} cultures compared to eASC\textsuperscript{WT} culture after 11 days (Fig. 2b). NO synthesis was also significantly increased in eASC\textsuperscript{NOS} compared to eASC\textsuperscript{WT} (Fig. 2c). Quantification of calcium deposition showed increased levels of calcium deposition in eASC\textsuperscript{NOS} compared to eASC\textsuperscript{WT} (Fig. 2d). Quantitative analysis of Runx2 (Fig. 2e) and ALP (Fig. 2f) gene expression were also significantly upregulated in the eASC\textsuperscript{NOS} cultures compared to the eASC\textsuperscript{WT} cultures.

NO-mediated osteogenic differentiation was further highlighted by inhibition of eNOS activity. eASC\textsuperscript{NOS} were treated with 2 mM of the nitric oxide synthase inhibitor, l-N\textsuperscript{3}-nitroarginine methyl ester (l-NNAME) for 11 days. l-NNAME treatment resulted in a significant downregulation of osteoblast-specific marker expressions, ALP (Fig. 3a) and Runx2 (Fig. 3b), compared to untreated eASC\textsuperscript{NOS}.

**Co-expression of eNOS and CAV-1\textsuperscript{PP2A} enhances NO production and osteogenic differentiation**

Lentiviral vectors expressing eNOS and CAV-1\textsuperscript{PP2A} (mutant) or CAV-1\textsuperscript{WT} (wild-type) were co-expressed in eASCs, eASC\textsuperscript{NOS+CAV-1\textsuperscript{PP2A}} and eASC\textsuperscript{NOS+CAV-1\textsuperscript{WT}}, respectively. Co-expression of eNOS and CAV-1\textsuperscript{PP2A} promoted osteogenesis as evident by Alizarin Red S staining (Fig. 2b) and calcium deposition (Fig. 2d) compared to eNOS alone (eASC\textsuperscript{NOS}), and NO levels were also significantly increased in the eASC\textsuperscript{NOS+CAV-1\textsuperscript{PP2A}} (Fig. 2c). Co-expression of eNOS with CAV-1\textsuperscript{WT} in eASCs (eASC\textsuperscript{NOS+CAV-1\textsuperscript{WT}}) reduced NO production (Fig. 2c) and also osteogenesis as evident by Alizarin Red staining (Fig. 2b) and calcium deposition (Fig. 2d). Quantitative real-time PCR analysis revealed that Runx2 (Fig. 2e) and ALP (Fig. 2f) were significantly upregulated in the eASC\textsuperscript{NOS+CAV-1\textsuperscript{PP2A}} cultures and downregulated in the eASC\textsuperscript{NOS+CAV-1\textsuperscript{WT}} cultures as compared with the eASC\textsuperscript{NOS} cultures (Fig. 2e and f).

**Exogenous NO donor enhances osteogenesis of eASC in a dose-dependent manner**

To confirm the direct role of NO levels on eASC osteogenesis, we treated eASCs with a concentration range of exogenous NO donor (NONOate; Sigma-Aldrich). Treatment with exogenous NO donor promoted osteogenesis from 5 \( \mu \)M to 15 \( \mu \)M but this was reduced with high concentrations of NO donor (20 \( \mu \)M) as evident by Alizarin Red staining (Fig. 4a). NONOate treatment also resulted in a dose-dependent increase in Runx2 (Fig. 4b) and ALP (Fig. 4c) gene expression, in which maximum levels of both were achieved with 15 \( \mu \)M NONOate; notably, significantly lower levels of Runx2 and ALP expression were observed with 20 \( \mu \)M NO donor treatment.
NO promotes endogenous Runx2 expression in differentiating eASCs

To monitor the effect of NO on endogenous Runx2 expression in differentiating eASCs, we generated a GFP lentiviral reporter system under the control of a Runx2 enhancer fused to the Hsp68 promoter (Runx2-Hsp68-eGFP; Fig. 5a) based on a novel Runx2 enhancer. eASCs which were stably transduced with the Runx2 reporter...
showed low levels of GFP expression in mostly undifferentiated cells (Fig. 5b), whereas GFP expression as a result of Runx2 promoter activity in differentiating eASCs was increased (Fig. 5b). When eASCs were transduced with eNOS (eASC\textsuperscript{eNOS}), the GFP signals were increased compared to eASCs co-transduced with eNOS and CAV-1\textsuperscript{WT} (eASC\textsuperscript{eNOS+CAV-1WT}) and un-transduced control (eASC\textsuperscript{WT}) (Fig. 5b). Interestingly, we observed that endogenous Runx2 activity was remarkably increased when the eASCs were co-transduced with eNOS and CAV-1\textsuperscript{FREA} (eASC\textsuperscript{eNOS+CAV-1FREA}) (Fig. 5b) compared to eASC\textsuperscript{eNOS}, eASC\textsuperscript{eNOS+CAV-1WT}, and eASC\textsuperscript{WT}. Osteogenic node
formation was significantly increased in the eASC
NOS
+CAV
+PRP
(Fig. 5c), and these result suggests that en
dogenous Runx2 expression is less active in undifferenti-
ed eASCs and its expression is significantly increased
through NO signaling during osteogenic differentia-
tion.

**NO modulates Wnt signaling to promote osteogenic
differentiation**

To examine the role of canonical and non-canonical Wnt
signaling during NO-mediated osteogenic differentiation,
expression of Wnt3a, Wnt8a, and Wnt5a was assessed
by quantitative real-time PCR. Non-canonical Wnt5a
expression was reduced in eASC
NOS
+CAV+PRP
(Fig. 6c), and was
significantly further decreased in eASC
NOS
+CAV+PRP+Ati
eASC
NOS
+CAV+PRP+Ati
(Fig. 6c). However, expression of canonical Wnt ligands
Wnt3a (Fig. 6a) and Wnt8a (Fig. 6b) was upregulated in
eASC
NOS
and significantly further increased in eASC
NOS
+CAV+PRP+Ati
(Fig. 6a and b, respectively). Treatment with 2 mM
NAME showed downregulation of Wnt5a expression
(Fig. 6d) and upregulation of Wnt5a (Fig. 6e) in eASC
NOS
, indicating that NO modulates Wnt signaling pathway
in eASCs.

Furthermore, treatment with NO donor (NONOate)
also resulted in increased expression of canonical Wnt
ligands Wnt3a (Fig. 7a) and Wnt8a (Fig. 7b), and down-
regulation of non-canonical Wnt5a expression (Fig. 7c) in
a dose-dependent manner from 5 μM to 15 μM of NON-
Oate. Interestingly, when the NO donor concentration
was increased up to 20 μM, the effect was completely re-
versed by downregulating Wnt3a (Fig. 7a) and Wnt8a
(Fig. 7b), and upregulating Wnt5a expression (Fig. 7c).
Control cells (eASCs in normal growth medium) also
showed increased expression of Wnt5a (Fig. 7c), suggest-
ing that induction of osteogenic differentiation of eASCs
requires activation of canonical Wnt signaling and sup-
pression of non-canonical Wnt5a expression.

To further analyze the relationship between NO-induced
osteogenic differentiation and Wnt signaling, eNOS-
transduced eASCs were treated with 20 ng/mL of the Wnt
signaling inhibitor Dickkopf-related protein 1 (Dkk-1).
Dkk-1 treatment resulted in a significant downregulation
of the osteoblast specific markers ALP (Fig. 8a) and Runx2
(Fig. 8b) compared to untreated eNOS transduced cells.

**Canonical Wnt3a promotes osteogenesis while
non-canonical Wnt5a suppresses osteogenesis**

To explore the opposite effects of canonical and non-
canonical Wnt signaling pathways on eASC osteogenesis,
we generated canonical Wnt3a and non-canonical Wnt5a expressing lentiviral vectors and transduced eASCs. Wnt3a-transduced eASCs (eASC WT;w3a) and Wnt5a-transduced eASCs (eASC WT;w5a) were incubated in osteogenic induction medium (OM) for 11 days. Interestingly, we found that Wnt3a (eASC WT;w3a) promoted osteogenesis as evident by Alizarin Red staining compared to untransduced eASCs (eASC WT;w+) (Fig. 9a). On the other hand, overexpression of Wnt5a (eASC WT;w5a) reduced osteogenic differentiation (Fig. 9a). Quantitative analysis of the mRNA levels by real-time PCR revealed that ALP (Fig. 9b) and Runx2 (Fig. 9c) were upregulated in the eASC WT;w3a culture as compared with the ASC WT culture, and downregulated in eASC WT;w5a culture, suggesting that lentiviral vector-mediated Wnt3a expression can promote osteogenesis while expression of non-canonical Wnt5a suppresses osteogenesis.

NO promotes the canonical Wnt signaling pathway by promoting nuclear translocation of β-catenin

To further explore mechanisms by which NO promotes canonical Wnt signaling, we used a lentiviral vector expressing GFP reporter under the control of the TCF/LEF promoter (TOPFLASH; Adgptn). In the canonical Wnt signaling pathway, β-catenin translocation to the nucleus is promoted by the activation of canonical Wnt signaling [52, 53]. Accordingly, eASCs were introduced with the lentiviral TCF/LEF-ΔGFP reporter, and those cells were then co-transduced with doxycycline inducible eNOS expressing lentiviral vector (Fig. 10a). As a readout for nuclear translocation of β-catenin, the TCF/LEF-driven GFP mRNA expression levels were measured by quantitative real-time PCR. Under osteogenic induction conditions, increased GFP mRNA expression was demonstrated compared to non-osteogenic induction...
conditions (cells in growth medium) (Fig. 10b). When doxycycline was added to the OM, GFP mRNA expression was significantly increased in the eASC-transduced cells (eASC-eNOS) (Fig. 10b). Interestingly, when doxycycline was removed from the medium, GFP mRNA expression in eASC-eNOS was similar to other osteogenic induction conditions (Fig. 10b).

Using a β-catenin-specific monoclonal antibody, we further investigated the effect of NO on β-catenin nuclear translocation. eASCs were transduced with the doxycycline
inducible eNOS lentiviral vector followed by immunostaining with β-catenin-specific monoclonal antibody (Cell Signaling Technology). When doxycycline was added to the CM, the expression of β-catenin was observed in eNOS-transduced cells (eASC\(^{\text{eNOS}}\)) (Fig. 11a) in both the nucleus and cytoplasm, and when doxycycline was removed from the culture, the expression of β-catenin in eASC\(^{\text{eNOS}}\) was reduced (Fig. 11a) to that in un-transduced control cells (Fig. 11a). Furthermore, we observed nuclear co-localization of β-catenin and DAPI only in doxycycline-treated eASC\(^{\text{eNOS}}\) suggesting that NO may promote nuclear localization of β-catenin (Fig. 11b). As a control for the primary antibody, immunostaining was carried out in the absence of the primary antibody specific to β-catenin (Additional file 2: Figure S2).

Together, these findings support the paradigm that cellular environments rich in bioavailable NO through either genetic modification or exogenous sources can modulate Wnt signaling, by upregulating the canonical and downregulating the non-canonical pathways resulting in increased osteogenic differentiation (Fig. 12).

**Discussion**

NO plays an important role in osteogenesis, bone remodeling, and metabolism [54–56]. It has been reported that both iNOS and eNOS play a role in osteogenesis of embryonic stem cells [57]. We [4] and others [58] have shown that MSCs do not express eNOS. Therefore, in order to investigate the role of eNOS in osteogenic differentiation of eASCs, in this study eASCs were genetically modified by lentiviral vector-based eNOS. ASCs are promising candidates for stem cell-based therapy for bone repair [59], and the role of eNOS-mediated NO synthesis and its downstream effects on osteogenesis of MSCs remains to be explored. We found that eNOS gene transfer by lentiviral vector promoted osteoblast-specific gene expressions (Fig. 2e and f), contributing to the matrix mineralization as visualized by Alizarin Red S staining (Fig. 2h and d). Noteworthy, this osteogenic potential of eASCs\(^{\text{eNOS}}\) was significantly abrogated by l-NAME treatment (Fig. 3), suggesting that NO derived from eNOS plays a major role in enhancing osteogenesis in eASCs.

Cav-1 is a key negative regulator of eNOS activation and thus inhibits the production of NO [41, 50], and it is important, Cav-1 is expressed endogenously in MSCs [61]. The scaffolding domain (82-101 amino acids) of Cav-1 protein interacts with eNOS at the plasma membrane and this interaction inhibits the eNOS activation reducing NO synthesis [41]. An alanine scanning approach revealed that substitution of phenylalanine at the amino acid position 92 with alanine to produce Cav-1\(^{92P} \)mutant restored the eNOS
Fig. 11 Nitric oxide promotes nuclear translocation of β-catenin. a Immunostaining with a β-catenin-specific monoclonal antibody reveals that the expression of β-catenin in endothelial nitric oxide synthase (eNOS) transduced cells where doxycycline (DOX) is available in the medium. b Nuclear localization of β-catenin in eNOS transduced cells in DOX-containing medium. Arrow indicates nuclear localization of β-catenin.

Fig. 12 Proposed signaling mechanism underlying osteogenic differentiation induced by NO in eNOS. Molecular control of NO levels may activate and suppress the expression of endogenous canonical and non-canonical Wnt ligands, respectively, to promote nuclear localization of β-catenin and subsequent activation of osteogenic differentiation through promoting osteoblast-specific gene transcription, CAV-1 WT, mutated cavedolin-1, CAV-1 WT, wild-type cavedolin-1, eNOS endothelial nitric oxide synthase.
activation and promoted NO synthesis [41]. Thus, in order to understand the contribution of Ror2 in the control of NO synthesis in eASC osteogenesis, we modified eASCs by expressing CAV-1WT (as a negative regulator for eNOS activation) or CAV-1β-catenin (as a positive regulator for eNOS activation) together with eNOS. Confirming our previous observation [41], we found that co-expression of eNOS and CAV-1β-catenin increased NO production while eNOS and CAV-1WT co-expressed eASCs showed reduced NO production ([Fig. 5c]), suggesting that CAV-1 is an important regulator of NO production in eASCs. We further found that these controlled levels of NO synthesis regulate osteogenesis, where eNOS together with CAV-1β-catenin resulted in increased osteogenic differentiation of eASCs.

To explore the molecular basis of NO-mediated osteogenesis, we investigated the effect of NO on Wnt signaling. Wnt signaling pathways have been shown to regulate osteoblastogenesis [62], in which canonical Wnt ligands promote osteogenesis [63, 64], and non-canonical Wnt signaling can inhibit the canonical Wnt signaling [65]. In the canonical Wnt pathway, binding of canonical Wnt ligands such as Wnt3a and Wnt8a to cell surface frizzled receptors results in the nuclear translocation of β-catenin [66], which ultimately binds with the TCF/LEF family to initiate the transcription of osteogenic genes such as Runx2 [62]. On the other hand, binding of non-canonical Wnt5a ligand to the ROR2 member of the Ror-family of RTKs inhibits canonical Wnt signaling by promoting β-catenin degradation, and downregulation of β-catenin reduced osteoblast-specific gene expression [67]. Our results revealed that genetic manipulation of eASCs with eNOS and CAV-1β-catenin increased canonical Wnt3a and Wnt8a expression, whereas eASCs transduced using eNOS/CAV-1WT decreased Wnt3a and Wnt8a expression ([Fig. 6a and b]), suggesting that NO levels may regulate Wnt ligand expression and promote osteogenesis. Confirming the role of Wnt signaling on osteogenesis, inhibition of canonical Wnt signaling through Dkk-1 treatment of eNOS-expressing cells attenuated osteogenesis as evident by downregulation of osteoblast-specific Runx2 and ALP expression ([Fig. 8]). On the other hand, the effect of non-canonical Wnt5a expression was completely the opposite ([Fig. 6c]) to the canonical Wnt3a and Wnt8a expression profiles, suggesting that molecular control of NO synthesis through eNOS/CAV-1 interaction or enogenous NO treatment ([Fig. 7]) results in differential regulation of Wnt ligand expression and their subsequent effect on osteogenic differentiation. Furthermore, we also found that NO modulates Wnt signaling and promotes osteogenesis when a differentiation environment is enhanced with an optimum concentration of oxygen NO (Figs. 4 and 7).

It has been shown that Wnt3a can directly promote osteogenesis [68], whilst Wnt5a plays a role in self-renewal of stem cells [69]. We further investigated the direct effect of canonical Wnt3a and non-canonical Wnt5a on eASC osteogenesis through lentiviral vector overexpression. Interestingly, concomitantly our results on NO-mediated Wnt-regulated osteogenesis, Wnt3a promoted osteogenesis ([Fig. 9a–c]) whereas Wnt5a inhibited osteogenesis ([Fig. 9a–c]). It was shown that increased levels of β-catenin can promote bone formation through increasing the expression of osteoblast-specific genes [70, 71], whilst abnormal osteoblast differentiation has been observed with β-catenin knockdown [70, 72]. Thus, it is possible that Wnt3a promotes osteogenesis by increasing β-catenin stability and Wnt5a may suppress osteogenesis by degrading β-catenin. NO may regulate this mechanism by increasing Wnt3a and suppressing Wnt5a ligand availability to modulate nuclear localization of β-catenin via the canonical Wnt ligand transduction pathway. In support of this, we observed that eNOS-transduced cells promoted the expression of β-catenin and its nuclear localization ([Fig. 11]), and a TCF/LEF-EVs reporter assay demonstrated responsiveness in a NO-rich cellular environment ([Fig. 10b]), which could be controlled through the expression of DOX-inducible eNOS.

Conclusions

In summary, our findings provide an insight into the role of NO in promoting eASC osteogenic differentiation in a cellular environment of optimum levels of NO through interaction with Wnt signaling pathways. This may lead to the development of novel cell-based therapeutic approaches for bone repair, in particular in vitro modification of MSCs by NO to optimize the endogenous Wnt signaling pathway to promote osteogenic differentiation upon subsequent transplantation.


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Appendix 4

Sequence information of 1049 bp long human FLT1 promoter

5’ TTTGCTTCTAGGAAGCAGAAGACTGAGAAATGACTTGGGGCGGAGGTGCATCAATGCACGCGGCAA
AAAAGAGACACGAGACACGCCCTCCCTGGGACCTGAGCTGGTTCGCAGTCTTCCCAAAGGGAGTGGCAA
GCAAGCGTCAGTTCCCCCTGAGCGCTCCAGTTGAGTTGCGCTTTGGTGCGGAGGCTTCCGGTGCC
CTCCTAGACCTTTCTGAGGACAGTTTGAGGCTAAGAGCGCGCGGAGGAGGAGGAGGAGGGAGGAG
CAGGCAAGGGAGACACGAGGACTGCCTTCAGCTCCTCCTCTCCTGAAGTAACCAGGTAGCGGGAG
GCCGCGGCGCCAGCTTCCCTGAGACTTGGAGCTTTGCGGCCCTAGGGCCAGGGCGGAGGCTTTCA
GCCTTTGTCCTCCCAGTTTCGGGCGGCGCCAGAGCTGAGTAAGCCGGGTGGAGGGAGTCC
CTGCAAGAGATTTCTTCGAGCGGCGATGGAAGGГАТGGГАГГГАГГГАГГГАГГГГАГГГГАГГГ
CAАAGАCCTGAACTCCTGCGGGCGGACCCGCGCTCCCGGGCCCGTCGCCAGCACCTCCCCACG
СGCGCTGCCGGCGGCACCGCGCATCGTGCGCGCCCGCCCGCCCCCTCTCCGTAGCCGCAGGG
AAGCGAGCCTGGGAGGAAGAAGAGGGTAGGTGGGGAGGCGGATGAGGGGTGGGGGACCCC
TTGAGTCACCAGAAGGAGGGTTGGGAGGTGGGCTGGGGAAAGGTTATAAATGTC
CCCGGCCTCGGCTCTCGATTGCGGGACGGCTCGGAGCGCAGGCGGACAC
TTCGCGGTCCCGCTCCTCCGCGGCGGCGGCGGCGGCGGCTCCGAGGCGGCTCGGCTCGGCT
AGCGCGAGGGCCGCGCTGGGAGGAAATGGTGGATTTGTCGCTGGCTGGGA
GCCGAGACGCGCGCTCAGGCGCGCGGCGGCGGCGGCGGCGGCGGACACGAGGAGGACTCTG
GCCGCGGCGTCTGGCGGCGGCGGAGGCGGCGGAGGCGGAGGCGGAGGCGGCGGCGGAGGCGGCGG
CACC 3’
Minicircle DNA-mediated endothelial nitric oxide synthase gene transfer enhances angiogenic responses of bone marrow-derived mesenchymal stem cells

Nadeeka Bandara3, Sallya Gurusinghe3, Haiying Chen2, Shuangfeng Chen2, Le-xin Wang22, Shiang Y. Lim34 and Padraig Strappe**

Abstract

Background: Non-viral-based gene modification of adult stem cells with endothelial nitric oxide synthase (eNOS) may enhance production of nitric oxide and promote angiogenesis. Nitric oxide (NO) derived from endothelial cells is a pleiotropic diffusible gas with positive effects on maintaining vascular tone and promoting wound healing and angiogenesis. Adult stem cells may enhance angiogenesis through expression of bioactive molecules, and their genetic modification to express eNOS may promote NO production and subsequent cellular responses.

Methods: Rat bone marrow-derived mesenchymal stem cells (RBMScs) were transfected with a minicircle DNA vector expressing either green fluorescent protein (GFP) or eNOS. Transfected cells were analysed for eNOS expression and NO production and for their ability to form in vitro capillary tubules and cell migration. Transcriptional activity of angiogenesis-associated genes, CD31, VEGF-A, PDGF-R, FGF-2, and FGF-R2, were analysed by quantitative polymerase chain reaction.

Results: Minicircle vectors expressing GFP (MC-GFP) were used to transfect HEK293T cells and RBMSCs, and were compared to a larger parental vector (P-GFP). MC-GFP showed significantly higher transfection in HEK293T cells (55.1 ± 3.3 %) and in RBMSC (18.65 ± 1.05 %) compared to P-GFP in HEK293T cells (43.4 ± 4.9 %) and RBMSC (15.21 ± 0.22 %). MCeNOS vectors showed higher transfection efficiency (21 ± 3 %) compared to P-eNOS (9 ± 1 %) and also generated higher NO levels. In vitro capillary tubule formation assays showed both MC-eNOS and P-eNOS gene-modified RBMSCs formed longer (14.66 ± 0.055 mm and 13.38 ± 0.68 mm, respectively) and a greater number of tubules (56.33 ± 3.51 and 51 ± 4, respectively) compared to controls, which was reduced with the NOS inhibitor L-NAME. In an in vitro wound healing assay, MC-eNOS-transfected cells showed greater migration which was also reversed by L-NAME treatment. Finally, gene expression analysis in MC-eNOS-transfected cells showed significant upregulation of the endothelial-specific marker CD31 and enhanced expression of VEGF-A and FGF-2 and their corresponding receptors PDGF-R and FGFR2, respectively.

Conclusions: A novel eNOS-expressing minicircle vector can efficiently transfect RBMSCs and produce sufficient NO to enhance in vitro models of capillary formation and cell migration with an accompanying upregulation of CD31, angiogenic growth factor, and receptor gene expression.

Keywords: Minicircle, DNA vector, Transfection, Endothelial nitric oxide synthase, Mesenchymal stem cells, Nitric oxide, Angiogenesis

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Background
Development of safe and efficient systems for gene transfer is required for translation of gene-modified stem cells into therapeutic applications. Conventional plasmid DNA (pDNA)-based non-viral vectors contain bacterial sequences and transcriptional units that may contribute to an immune response against bacterial proteins expressed from cryptic upstream eukaryotic expression signals. Furthermore, changes in eukaryotic gene expression may be altered due to the antibiotic resistance marker and immune responses to bacterial CpG sequences [1]. These prokaryotic DNA sequences present in pDNA vectors may lower their biocompatibility and safety. In clinical studies, unmethylated CpG motifs induced inflammatory responses [2] and necrosis- or apoptosis-mediated cell death in target cells, resulting in short-lived transgene expression [3, 4]. Furthermore, during the intracellular trafficking of pDNA, the bacterial sequences of pDNA vectors are rapidly associated with histone proteins, packing the sequences into a dense heterochromatin structure. If these are spread into the adjacent transgene in the vector, the sequences can become inaccessible by transcription factors, leading to reduced transgene expression through silencing of the eukaryotic promoter [5]. The removal of CpG islands by cloning out, or elimination of non-essential sequences, can reduce these undesirable responses but is time-consuming and tedious.

Minicircle (MC) pDNA technology consists of supercoiled DNA molecules for non-viral gene transfer, which has neither a bacterial origin of replication nor an antibiotic resistance gene [6]. MCs can be generated in E. coli by attachment sites (attP and attB), with specific recombination mediated by the phage P33I integrase [1]. As a result of this recombination event between attP and attB sites, MCs contain only a eukaryotic expression cassette and the attR fragments are formed but are devoid of bacterial backbone sequences. Absence of the bacterial backbone sequences leads to a size reduction in the MC relative to the parental pDNA which can enhance in vitro transfection efficiency [7] and in vivo gene delivery [8, 9]. Gene expression from non-viral episomal vectors may also enhace persistence of transgene expression without interrupting to the cellular genome [10].

Endothelial nitric oxide synthase (eNOS), also known as NOS3, is expressed in endothelial cells [11], and is responsible for generating nitric oxide (NO) which plays an important role in vasculogenesis [12, 13]. NO produced from endothelial cells is important for maintaining vascular integrity and may enhance vasculogenesis through fibroblast growth factor (FGF) signalling [14]. Vascular endothelial growth factor (VEGF) is also induced by the NO synthesis pathway [15] contributing to angiogenesis. eNOS knockout mice (eNOS-/-) display impaired vasculogenesis [16] and have also demonstrated diminished wound healing due to reduced VEGF-mediated migration of endothelial cells [17] and bone marrow progenitor cells [18] to the sites of injury. eNOS-based gene therapy approaches have shown restoration of impaired angiogenesis in rats [19, 20] and promotion of re-endothelialisation [21] in injured rabbits upon adenovirus-mediated eNOS gene transfer.

Similar to endothelial progenitor cells, mesenchymal stem cells (MSCs) also participate in post-natal angiogenesis [22], and vascular pericytes, which are crucial for maintaining vascular integrity, share similar phenotypic features with MSCs [23]. Exogenously administered, MSCs form new capillaries and medium-sized arteries [24] which are important properties of tissue regeneration by MSCs [25]. MSCs can differentiate into endothelial cells in vitro [26] and contribute to neovascularisation, particularly during tissue ischaemia and tumour vascularisation [27]. In MSCs, VEGF-A binds with platelet-derived growth factor receptor (PDGFR) to initiate VEGF-A/PDGFR signalling and drive vasculogenesis, as opposed to the VEGFR2 in endothelial cells, which is absent on MSCs [28]. NO has been shown to upregulate PDGFRa receptor expression in rat mesangial cells [29], and the induction of tumour angiogenesis has been linked to the NO-induced Notch signalling pathway in PDGFR-activated mouse glioma cells [30]. FGFR2 signalling also enhances vasculogenesis through promotion of NO production [31, 32]. eNOS is the only NOS isoform absent in MSCs [33], and hence eNOS-based genetic modification of MSCs may enhance their therapeutic application. In this study, we describe a novel non-viral MC vector to deliver the eNOS transgene to MSCs with higher transfection efficiency than regular plasmids. NO signalling in the gene-modified MSC promotes capillary tube-like network formation and cell motility. Quantitative real time polymerase chain reaction (PCR) data revealed that MC-mediated eNOS gene transfer significantly upregulates endothelial-specific CD31 gene expression. Furthermore, NO upregulates the angiogenic responsive genes VEGF-A and FGFR2 and expression of their corresponding receptors, PDGFRa and FGFR2.

Methods
Rat bone marrow-derived mesenchymal stem cell isolation
All experiments involving animals were approved by the Charles Sturt University animal ethics committee. MSCs were isolated from the bone marrow of 8–12 week old male Sprague–Dawley rats as previously described [33].

Tri-lineage differentiation of rat bone marrow-derived mesenchymal stem cells
The ability of the isolated rat bone marrow-derived MSCs (rBMSCs) (Passage 6) to differentiate to adipogenic,
osteogenic and chondrogenic lineages was investigated. To induce osteogenic differentiation, rBMSCs at 80–90 % confluency were incubated in osteogenic-defined medium (Dulbecco’s modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 10 mM beta-glycerol phosphate, 10 mM dexamethasone and 0.2 mM L-ascorbic acid 2-phosphate) for 11 days with medium changed twice a week, as described previously [34]. Cells were then fixed with 4 % paraformaldehyde and stained with Alizarin Red S (pH 4.1) as described previously [35].

To induce adipogenic differentiation, rBMSCs at 80–90 % confluency were incubated in adipogenic-defined medium (DMEM supplemented with 10 % FBS, 10 μM indomethacin, 1 μM dexamethasone, 0.8 μM insulin, 0.5 mM resiglitazone) [36] for 1 week with media changed twice. Adipogenic differentiation was assessed by 0.18 % Oil Red O staining after fixing the cells in 10 % neutral-buffered formalin (NBF) [35].

To induce chondrogenic differentiation, three-dimensional pellet cultures of rBMSCs (2.5 × 10^5 cells) were formed by centrifugation at 500 × g in 10 ml conical-bottomed sterile tubes. The chondrogenic induction medium consisted of DMEM supplemented with 1 x ITS + 3 (Sigma), 1 x non-essential amino acids (Sigma), 10 ng/ml transforming growth factor β (TGF-β; Peprotech), 100 nM dexamethasone, and 2 μM ascorbic acid (Sigma) [37]. Pellet cultures were incubated in induction medium for 14 days with the medium changed every second day with the lids of the tube loosened to facilitate gas exchange. At day 14 the pellets were fixed in 10 % NBF for 24 h, and the three-dimensional tissues were processed and embedded in paraffin wax for micrometre processing. To assess chondrogenic differentiation, embedded pellets were sectioned (5 μm slices) and stained with 1 % Alcan blue to visualise glycosaminoglycan accumulation.

The images for differentiated cells into all three lineages were captured by a colour camera (Nikon Digital Sight Ds-Fi2) attached to a Nikon Eclipse-Ti-U microscope (Nikon).

Production of minicircle plasmid DNA-expressing eNOS
To construct an eNOS expressing minicircle vector, a codon optimized human eNOS cDNA sequence (3633 bp) was cloned into the minicircle parental plasmid consisting of expression cassette CMV–MCS–EF1α–GFP–SV40–PolyA (P-GFP) (System Biosciences, Mountain View, CA, USA). This cloning strategy allowed removal of the EF1α–GFP portion from the final construct (P-eNOS).

The minicircle DNA plasmids expressing eNOS and GFP were produced according to the manufacturer’s instructions (System Biosciences). Briefly, E. coli ZYCY710PS2T cells were transformed with P-GFP and P-eNOS. Following this, single colonies were grown in 2 ml LB (luria broth) media containing 50 μg/ml kanamycin for 1 h at 30 °C with vigorous shaking at 200 rpm. Next, 50 μl of the starter culture was then used to inoculate 200 ml fresh terrific broth (TB; Sigma) in a 1 litre flask with 50 μg/ml kanamycin followed by incubation at 30 °C for 17 h with constant shaking at 200 rpm. Minicircle induction medium consisting of 200 ml LB (luria broth), 8 ml 1 N NaOH and 200 μl 20 % L-arabinose was combined with the TB bacterial culture and incubated for a further 4 h at 30 °C with constant shaking at 200 rpm. Minicircle plasmid DNA (MC-eNOS and MC-GFP) was isolated using a Genomed Jetstar 2.0 midi kit according to the manufacturer’s instructions (Genomed, Germany) and treated with plasmid-safe ATP-dependent DNase (Epigen, USA) to remove bacterial genomic DNA contamination. eNOS- and GFP-containing minicircles were designated as MC-eNOS and MC-GFP, respectively.

Cell culture and transfection
Human embryonic kidney (HEK293T) cells and rBMSCs were maintained in DMEM (Sigma) supplemented with 10 % (v/v) FBS (Sigma), 1 % (v/v) L-glutamate (Sigma) and 1 % (v/v) penicillin/streptomycin antibiotics mix (Sigma). Cells were transfected with the plasmids (P-GFP, MC-GFP, P-eNOS and MC-eNOS) using Lipofectamine 2000 reagent (Life technologies, USA) following the manufacturer’s instructions. GFP expression was assessed by fluorescence microscopy at 24 and 48 h after transfection, and flow cytometry analysis (Gallios Instrument, Beckmann).

Immunocytochemistry
Immunocytochemical detection of eNOS expression in P-eNOS and MC-eNOS transfected HEK293T and rBMSCs was performed as follows. Briefly, cells were fixed in 4 % paraformaldehyde for 20 min at room temperature, treated with 0.1 % Triton-X100 in phosphate-buffered saline (PBS) for 10 min, and blocked in a 10 % FBS in PBS solution for 30 min at room temperature. This was followed by a 2-h incubation with a primary mouse monoclonal anti-eNOS antibody (BD Bioscience), and subsequently with an anti-mouse IgG secondary antibody conjugated with Alexa 488 (Cell Signalling Technology) for 1 h followed by DAPI (nuclear stain) and phalloidin-TRITC (cytoskeleton stain) (Sigma). eNOS-positive cells were counted by fluorescence microscopy in five randomly selected fields per well in three independent experiments and 500–1000 cells were counted in total; the percentage of eNOS positivity was calculated from the total nuclear stained cells.
Nitric oxide detection

Nitric oxide released from P-eNOS and MC-eNOS transfected cells in cell supernatants was measured using the Griess reagent (Promega) following the manufacturer's instructions. NO was also directly detected in transfected cells using a specific fluorescent NO indicator, 4,5-diaminofluorescein diacetate (DAF-2DA; Cayman Chemicals, USA), as described previously [13, 38]. Cells were grown to confluence on a 12-well plate and incubated for 30 min with 1 μM DAF-2DA. Subsequently, cells were washed with fresh PBS and viewed by a fluorescence microscope.

In vitro angiogenesis

In vitro capillary formation was performed as described previously [37]. Briefly, Golitex-2 (Life technologies) was thawed on ice overnight and applied evenly over each well (50 μl) of a 96-well plate and incubated for 30 min at 37°C allowing polymerization. Transfected rBMSCs or control cells were seeded at 20,000 cells per well and grown in 100 μl angiogenic induction medium (DMEM [Sigma], 1.5% FBS, 1% (v/v) L-glutamate [Sigma] and 1% (v/v) penicillin/streptomycin [Sigma]) and incubated at 37°C for 5 h. The capillary network was fixed with 4% paraformaldehyde and visualized by staining with DAPI and Phalloidin (Sigma). The efficiency of in vitro tubule formation was evaluated by measuring the number of nodes and length of the tubules as described previously [13].

In vitro scratch wound healing assay

The effect of nitric oxide on cell migration was assessed using an in vitro scratch wound healing assay as described previously [37]. Briefly, HEK293T cells and rBMSCs were transfected with P-eNOS, MC-eNOS, P-GFP and MC-GFP in 6-well tissue culture plates.Next, 48 h following the transfection when the cells reached 100% confluence, scratch wounds were made using a sterile 200 μl pipette tip and the boundaries were marked. The cells were then cultured with 2 ml fresh DMEM supplemented with 10% (v/v) FBS (Sigma), 1% (v/v), L-glutamate (Sigma), and 1% (v/v) penicillin/streptomycin (Sigma). Phase-contrast microscopy images were acquired at 0 and 1 h after scratches were created for rBMSCs and after 17 h for HEK293T cells. Cell migration was measured at the indicated times by measuring the distance from the initial boundary edge to the boundary of the migrating cells, followed by calculation of the percentage of wound closure as follows: percentage of wound closure = (distance from the boundary edge at 0 h – distance from the boundary edge at 1 h or 17 h)/ (distance from the boundary edge at 0 h) × 100.

Gene expression by quantitative real time PCR

Total RNA from transfected and control cells was isolated using the PureZol reagent (BioRad) according to the manufacturer's instructions and the concentration of isolated RNA was determined using a Nanodrop spectrophotometer (Thermo Scientific) following treatment with RQ1 RNase free DNase (Promega) to remove contaminating DNA. Then, cDNA was synthesized with 1 μg RNA using a High Capacity Reverse Transcription Kit (Life technologies). The quantitative real time PCR assays were performed on a BioRad CFX96 Real-Time system (BioRad) using the SoFast EvaGreen Supermix (BioRad). Primers used for target amplification are described in Table 1. Assays were performed in triplicate, and target mRNA expression was normalized to rat GAPDH mRNA levels using the ΔΔCt method.

Western blot analysis

Transfected and control cells were washed with ice-cold PBS (Sigma) twice, and lysates were prepared by homogenization of cells in RIPA buffer (Sigma), following mixing with 4× NuPAGE LDS sample buffer (Life technologies) and lysed by heating for 10 min at 70°C. Total proteins were separated by 4–12% Bis-Tris NuPAGE (Novex, Life technologies) and transferred to PVDF membrane (Millipore). After blocking with Odyssey blocking buffer (LI-COR) for 30 min at room temperature, the membrane was incubated with primary antibodies specific to eNOS (1:1000 dilution) and β-actin (LI-COR; 1:1000 dilution) overnight at 4°C. The membrane was washed with 0.1% tween in PBS three times for 10 min each, incubated with donkey
anti-rabbit IgG (H&L) (Alexa Fluor® 680) secondary antibody (Life Technologies; 1:20,000) at room temperature for 1 h, and antibody-bound proteins were visualized by fluorescence detection with a LI-COR odyssey system.

**Statistical analysis**

All experiments were performed in triplicate and at least three times and data analysed by an independent two-tailed Student’s t test. A p value <0.05 was regarded as statistically significant.
Results
Characterisation of rBMSCs
rBMSCs were isolated from adult Sprague-Dawley rats as previously described [33], and plastic adherent rBMSCs displayed typical fibroblastic morphology (Fig. 1a) [40]. Tri-lineage differentiation of the rBMSCs was performed in the appropriate media to osteoblasts as demonstrated by Alizarin Red S staining of mineralised extracellular matrix (Fig. 1b), to chondrocytes by Alcian Blue staining of proteoglycans in three-dimensional pellet cultures (Fig. 1c) and to adipocytes as shown by Oil Red O staining of lipid vesicles (Fig. 1d).

Transfection of P-GFP and MC-GFP vectors
The GFP expressing minicircle vector (MC-GFP) was produced from the parental plasmid (P-GFP) as described in the manufacturer’s instructions (Systems Bioscience). We observed an approximate 4-kb reduction in plasmid size following minicircle induction using L-arabinose (Fig. 2a). HEK293T cells and rBMSCs were transfected with a range of plasmid DNA concentrations (1 μg, 0.5 μg, 0.25 μg, 0.125 μg, 0.0625 μg). After 48 h post-transfection, the cells were visualised by fluorescence microscopy and analysed by flow cytometry to estimate the percentage of GFP-expressing (GFP+) cells (Fig. 2b). The optimum plasmid DNA concentration for transfection was 0.5 μg which showed highest transfection efficiency for both P-GFP and MC-GFP in both HEK293T (Fig. 2c) and rBMSC (Fig. 2d) cell types.

Transfection of HEK293T cells with MC-GFP plasmid resulted in a significantly higher number of GFP+ cells (55.51 ± 3.3 %) compared to P-GFP (43.4 ± 4.9 %). A similar trend was seen in rBMSCs, with MC-GFP resulting in a higher transfection efficiency (18.65 ± 10.5 %) compared to P-GFP (15.21 ± 0.22 %).

Generation of eNOS minicircle vector
To generate an eNOS minicircle expression plasmid vector, a codon optimized cDNA of human eNOS (3633 bp) was synthesised (Genart) and sub-cloned into the parental plasmid P-GFP (CMV-MCS-EF1-
GFP-SV40PolyA) (System Biosciences, Mountain View, CA, USA) at the BamHI and SalI restriction sites in the multiple cloning sites downstream to the CMV promoter resulting in removal of the EF1α promoter and eGFP coding sequence (Fig. 3a). The eNOS minicircle vector was constructed as described above for the MC-GFP vector. The cloning was confirmed by double digestion of the parental plasmid encoding eNOS (P-eNOS) with BamHI and SalI yielding a fragment of ~3.7 kb (Fig. 3b). A reduction of the P-eNOS vector size was also observed after the production of MC-eNOS, to approximately 5 kb (Fig. 3c).

Transfection of P-eNOS and MC-eNOS vectors
Transfection of HEK293T cells with P-eNOS and MC-eNOS was assessed by immunofluorescence staining (Fig. 4a) and western blot analysis (Fig. 4b), using an eNOS-specific monoclonal antibody (BD bioscience). Nitric oxide production from transfected cells was measured by the production of nitrite at 24 h and 48 h post-transfection and in un-transfected HEK293T cells (Fig. 4c). Both P-eNOS and MC-eNOS transfected HEK293T cells showed significantly higher nitrite accumulation in cell culture media (at both 24 h and 48 h) compared to P-GFP, MC-GFP transfected cells and un-transfected HEK293T controls. At 24 h post-transfection, HEK293T cells transfected with P-eNOS and MC-eNOS resulted in 3.8 ± 0.2 μM and 4.16 ± 0.12 μM nitrite concentrations, respectively (Fig. 4c). The NO production increased significantly at 48 h post-transfection, resulting in 4.18 ± 0.12 μM and 5.06 ± 0.13 μM for P-eNOS and MC-eNOS, respectively. Furthermore, detection of nitric oxide produced from transfected cells was also confirmed by DAF-2DA staining in live cells. Both P-eNOS and MC-eNOS transfected HEK293T cells emitted a strong green fluorescence signal compared to no fluorescence in un-transfected cells (Fig. 4d).

eNOS gene transfer to rBMSCs
Transfection of P-eNOS and MC-eNOS vectors into rBMSCs was confirmed by immunostaining (Fig. 5a), and western blot analysis (Fig. 5c) with an eNOS-specific monoclonal antibody (BD bioscience). Both
the assays confirmed that no endogenous eNOS expression was seen in un-transfected rBMSCs (Fig. 5a and c). Significantly higher transfection efficiency for MC-eNOS (21 ± 3 %) compared to P-eNOS (9 ± 3 %) (Fig. 5b) was observed which resulted in higher NO production for MC-eNOS transfected rBMSCs (1.93 ± 0.06 μM) than P-eNOS (1.78 ± 0.1 μM) (Fig. 5d) compared to controls after 24 h of transfection. NO production increased further in MC-eNOS transfected rBMSCs (2.20 ± 0.08 μM) compared to P-eNOS at 48 h post-transfection (1.84 ± 0.1 μM) (Fig. 5d). NO synthesis in transfected rBMSCs was also demonstrated DAF-2DA staining in both P-eNOS and MC-eNOS transfected rBMSCs (Fig. 5e).

**eNOS gene delivery enhances in vitro capillary tubule formation**

Rat BMSCs were transfected with 0.5 μg P-eNOS, MC-eNOS, P-GFP, and MC-GFP. Un-transfected rBMSCs were used as a control. Transfected cells were then plated on a 96-well cell culture plate coated with an extracellular matrix (Geltrex). Both MC-eNOS and P-eNOS transfected rBMSCs formed significantly longer (14.66 ± 0.55 mm and 13.38 ± 0.68 mm, respectively) tubules and a greater number of tubules (56.33 ± 3.51 and 51 ± 4, respectively) compared to rBMSCs transfected with P-GFP, MC-GFP and non-transfected cells (Fig. 6).

To confirm that capillary-like tubule formation was NO-mediated, eNOS transfected rBMSCs were treated with 2 mM of the nitric oxide synthase inhibitor, L-N^δ^-nitroarginine methyl ester (L-NAME). L-NAME treatment resulted in a significant impairment of the tubule network, in terms of length (7.33 ± 1.03 mm and 7.06 ± 0.88 mm for MC-eNOS and P-eNOS, respectively) and tubule number (24 ± 4 and 24 ± 2 for MC-eNOS and P-eNOS, respectively) compared to untreated cells (Fig. 6).

**Nitric oxide promotes in vitro cell migration**

Using the scratch wound healing assay [37], migration of eNOS transfected rBMSCs was assessed. Transfection of
P-eNOS and MC-eNOS enhanced cell migration compared to P-GFP, MC-GFP and un-transfected rBMSCs (MC-eNOS, 44.05 ± 0.81 % P-eNOS, 43.13 ± 3.45 % MC-GFP, 10.43 ± 2.63 % P-GFP, 11.39 ± 3.03 % and rMSC, 9.46 ± 4.13 %) (Fig. 7a and c). However, cell migration rates between P-eNOS and MC-eNOS were not significantly different. Inhibition of NO production by treatment with 2 mM L-NAME significantly diminished the cell migration rates of both MC-eNOS and P-eNOS transfected cells (12.18 ± 1.67 % and 15.59 ± 4.69 %, respectively) (Fig. 7a and c). Cell migration rates were not significantly different among MC-GFP, P-GFP and un-transfected cells (Fig. 7a and c). A similar phenomenon was observed with HEK293T cells (Fig. 7b and d).

**MC-eNOS gene transfer to rBMSCs induces endothelial CD31 gene expression**

We found a significant increase in CD31 mRNA expression by 0.42-fold in P-eNOS transfected cells compared to P-GFP, MC-GFP and un-transfected control, suggesting that eNOS gene transfer may promote endothelial differentiation of rBMSCs (Fig. 8). Interestingly, minicircle-mediated eNOS (MC-eNOS) gene transfer showed a highly significant increase in CD31 mRNA expression by 1.8-fold compared to P-eNOS, P-GFP, MC-GFP, and un-transfected control (Fig. 8). Treatment with 2 mM L-NAME abolished the CD31 expression (Fig. 8), suggesting that expression of endothelial CD31 in rBMSCs through eNOS gene transfer is NO-mediated.
**NO modulates VEGF-A/PDGFR and FGF2/FGFR2 signalling pathways in eNOS transfected rBMSCs**

Expression of two key genes, VEGF-A and FGF2, which are involved in angiogenesis and cell migration were examined by quantitative real time PCR. Upregulation of both VEGF-A by 1.19-fold and 1.0-fold in MC-eNOS and P-eNOS modified rBMSCs, respectively (Fig. 9a), and FGF2 by 1.08-fold in MC-eNOS and 0.74-fold in P-eNOS delivered rBMSCs (Fig. 9b), compared to P-GFP. MC-GFP delivered rBMSCs and un-transfected rBMSCs. Treatment with 2 mM L-NAME reduced both VEGF-A (Fig. 9a) and FGF2 (Fig. 9b) expression in P-eNOS and MC-eNOS transfected cells. Furthermore, delivery of P-GFP and MC-GFP did not affect the VEGF-A and FGF2 expression compared to control rBMSCs (Fig. 9a and 9b). Next, we examined the effect of NO on the expression of PDGFRA and FGFR2 receptors as they are corresponding receptors of VEGF-A and FGF2. Expression of PDGFRA was increased by 1.82-fold and 1.56-fold in MC-eNOS and P-eNOS transfected rBMSCs, respectively (Fig. 9c), and FGFR2 receptor expression was increased by 1.46-fold in MC-eNOS and 1.14-fold in P-eNOS delivered rBMSCs (Fig. 9d), compared to P-GFP. MC-GFP delivered rBMSCs and un-transfected rBMSCs. Treatment with 2 mM L-NAME abolished both the PDGFRA (Fig. 9c) and FGFR2 (Fig. 9d) expression in P-eNOS and MC-eNOS transfected cells. Furthermore, neither PDGFRA nor FGFR2 receptor expression were affected by the delivery of P-GFP and MC-GFP compared to control rBMSCs (Fig. 9c and d).

**Discussion**

Minicircle vectors are supercoiled DNA molecules that are devoid of bacterial backbone sequences such as a bacterial origin of replication, antibiotic resistance gene and CpG motifs [41], and primarily consist of a eukaryotic expression cassette [6]. Compared to conventional plasmid DNA, minicircle vectors benefit from higher transfection efficiencies and longer transgene expression, possibly attributed to a lower activation of gene silencing mechanisms [42].
In this study, minicircles expressing GFP exhibited higher in vitro gene transfer efficiency than the parental plasmid to both HEK293T cells and rBMSCs (Fig. 3). As expected transfection efficiency was higher in the transformed cell line (HEK293T) compared to primary rBMSCs. eNOS expressing minicircles also showed higher gene transfer efficiency than P-eNOS (Fig. 5b). This higher gene transfer efficiency may also account for the significantly increased level of NO synthesis by MC-eNOS compared to P-eNOS (Figs. 4 and 5). We reasoned that this high level of NO synthesis from MC-eNOS transfected rBMSCs may be attributed to the removal of other plasmid sequences, which can affect gene expression [42], and the smaller size of the minicircle may also provide a more efficient route to the nucleus for transcription. This process involves several steps, including cellular entry of DNA through the cell membrane, DNA diffusion into the cytoplasm, and DNA entry to the nucleus [43]. Importantly, the DNA diffusion step depends on the physicochemical properties of DNA such as its diffusion coefficient which is inversely proportional to its molecular weight [44, 45]. Endocytosis is a major route for entry of DNA–cationic lipid complexes through the cell membrane in vitro [46] which takes place following specific interactions between DNA and caveolae [47]. This mechanism is also limited by particle size, where larger DNA–cationic lipid complexes are not efficiently taken up by the endocytosis [47]. DNA uptake and transfer to the nucleus via the nuclear membrane results in successful gene transfer [48]. Minicircle plasmid vectors can overcome these cellular obstacles more efficiently and, combined with a lack of bacterial backbone sequences, reduced promoter methylation [49] may also contribute to the higher levels of gene transfer compared to larger parental plasmids.

Angiogenesis is a complex process involving endothelial cell proliferation and migration, remodelling of extracellular matrix, and tubular structure formation. These processes are tightly regulated by the actions of angiogenic cytokines such as VEGF-A and FGF [31]. Angiogenesis also requires endothelial cell-to-cell, and cell-to-matrix interactions, which are mediated by various cell adhesion molecules [50]. eNOS plays a key role in angiogenesis mediated by substance P, a potent endothelium-dependent vasodilator (NO releaser) [51]. It has also been demonstrated that eNOS-KO (knockout) mice show impaired angiogenesis [52].

NO has been shown to play an important role in angiogenesis both in vitro and in vivo, and furthermore NO also contributes to endothelial cell migration in vitro [52]. We found that eNOS gene transfer by MC vector remarkably promoted endothelial-specific CDS1 gene expression (Fig. 8), contributing to the capillary-like tubule network formation by rBMSCs (Fig. 6) and enhanced cell motility as evident by in vitro wound healing assay (Fig. 7). Noteworthy, in these assays, CDS1 mRNA expression, tubule formation and cell migration in transfected cells were significantly abrogated by L-NAME treatment, suggesting NO plays a major role in enhancing endothelial characteristics in rBMSCs. Collectively, our data may suggest that MC-mediated eNOS gene transfer may contribute to the reprogramming of adult stem cells into endothelial cells, which may be used in cell therapy applications involving vascular repair. Interestingly, Gomes and co-workers demonstrated that MSCs from S-nitrosoglutathione reductase (GSNOR)-deficient mice, where NO is produced mainly from iNOS (NOS2) rather than eNOS, exhibited attenuated vasculogenesis both in vitro and in vivo [13]. Furthermore, they revealed that pharmacological inhibition of NO in GSNOR−/− MSCs, or genetic reduction of NO
production in the NOS2−/−, enhanced vasculogenesis by MSCs than that for HUVECs, where NO synthesis is driven by eNOS enhanced vascular tube formation. MSCs have not been shown to express endogenous eNOS, unlike endothelial cells [53], and have been shown to participate in pro-angiogenic signalling [54]. Additionally, eNOS plays an important role in endothelial cell-mediated postnatal angiogenesis and vascular tone [55, 56]. NO may contribute to angiogenesis through VEGF and FGF signalling through an angiogenic switch which is preceded by a local increase in VEGF-A and FGF [31].
Nitric oxide can mediate the production of VEGF-A in human adipose-derived stem cells [57], and NO and FGF2 have also been shown to enhance angiogenesis in mouse embryonic stem cells [58]. Furthermore, FGF2 has been shown to induce eNOS expression [32]. Our data proposes that NO signalling through VEGF-A/PDGFrα and FGF2/FGFR2 pathways may directly promote rBMSC vasculogenesis (Fig. 9). We showed that eNOS transfected rBMSCs express increased levels of VEGF-A and FGF2 (Fig. 9) and their corresponding receptors PDGFrα and FGFR2, respectively (Fig. 9). It is noteworthy that MC-eNOS vector transfection was associated with a significantly higher FGF2 expression compared to the P-eNOS vector. Interestingly, treatment with L-NAME diminished the VEGF-A, PDGFrα, FGF2 and FGFR2 expression levels (Fig. 9) which were observed as being linked to impaired capillary tube-like network formation (Fig. 6). It has been shown that VEGF-A contributes to differentiation of MSCs to endothelial-like cells when co-cultured with endothelial cells expressing eNOS and this process is inhibited by VEGF-A antisera [59].

Angiogenesis is also associated with endothelial cell migration and proliferation [60]. Our results show that eNOS gene transfer into HEK293T and rBMSCs (Fig. 7) can increase cell motility compared to controls, and the effect is diminished by L-NAME treatment, suggesting that NO plays a role in regulating rBMSC cell migration (Fig. 7) which has been previously demonstrated for endothelial cell migration [61]. Together, these findings show that genetic manipulation of MSCs to enhance bioavailable NO may upregulate VEGF-A/PDGFrα and FGF2/FGFR2 signalling pathways to promote angiogenesis (Fig. 10).

Conclusions
In summary, this study demonstrates that NO derived from a minicircle DNA vector expressing eNOS exerts a positive effect on rBMSCs by promoting in vitro capillary tubule formation and cell migration and significant increases in angiogenesis-related gene expression. Use of MC-eNOS-based vectors may represent an efficient approach to gene therapy applications where enhancing NO bioavailability is beneficial.

Abbreviations
CAMS-DA: 4′,6-diamidino-2-phenylindole; DMEM: Dulbecco’s modified Eagle medium; eNOS: endothelial nitric oxide synthase; FBS: fetal bovine serum; FGF2: fibroblast growth factor (receptor); GFP: green fluorescent protein; LIF: leukemia inhibitory factor; L-NAME: 1-NAME: nitroarginine methyl ester; MC: mesenchymal MSC; mesenchymal stem cell; NBS: neutral buffered saline; NO: nitric oxide; PCR: polymerase chain reaction; PDGFrα: platelet-derived growth factor receptor; pDNA: plasmid DNA; rBMSC: rat bone marrow derived mesenchymal stem cell; TB: tendon broth; VEGF2: vascular endothelial growth factor (receptor).
Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
All conceived of the study, collected data, performed data analysis, and prepared the manuscript. SC collected data and performed data analysis and prepared the manuscript. HC and SC collected data and prepared the manuscript. YL performed data analysis and prepared the manuscript. SY conceived of the study, and prepared and revised the manuscript. All authors read and approved the final manuscript.

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