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Role of KLF4 in regulation of myocardin induced SMC differentiation in human smooth muscle stem progenitor cells (hSMSPC)

Kalaimathi Govindarajan MSc

A thesis submitted in fulfilment
of the requirement for the degree of
Doctor of Philosophy

National University of Ireland
Centre for Research in Vascular Biology

April 2013

Supervisor:
Prof. Noel Caplice MD PhD

Declaration

I hereby declare that the research presented in this thesis is my own work and effort and that it has not been submitted for any other degree, either at University College Cork or elsewhere. Wherever contributions of others are involved, they have been acknowledged.

The work was done under the guidance of Professor Noel Caplice MD PhD at the Centre for Research in Vascular Biology, University College Cork.

Signature.....

Date.....

Abstract

The differentiation of stem cells into multiple lineages has been explored in vascular regenerative medicine and tissue engineering. However, in the case of smooth muscle cells (SMC), issues exist concerning inefficient rates of differentiation and a lack of understanding of the molecular mechanisms underpinning smooth muscle phenotype development. In stem cells, multiple repressors potentially downregulate myocardin, the potent SRF coactivator induced SMC transcription including Krüppel like zinc finger transcription factor-4 *(*KLF4). This thesis aimed to explore the role of KLF4 in the regulation of myocardin gene expression in human smooth muscle stem/progenitor cells (hSMSPC), a novel circulating stem cell identified in our laboratory which expresses low levels of myocardin and higher levels of KLF4*.* hSMSPC cells cultured in SmGM2 1% FBS with TGF-β1 (5 ng/ml "differentiation media") show limited SMC cell differentiation potential. Furthermore, myocardin transduced hSMSPC cells cultured in differentiation media induced myofilamentous SMC like cells with expression of SM markers. Five potential KLF4 binding sites were identified *in silico* within 3.9Kb upstream of the translational start site of the human myocardin promoter. Chromatin immunoprecipitation assays verified that endogenous KLF4 binds the human myocardin promoter at -3702bp with respect to the translation start site (-1). Transduction of lentiviral vectors encoding either myocardin cDNA (LV_myocardin) or KLF4 targeting shRNA (LV_shKLF4 B) induced human myocardin promoter activity in hSMSPCs. Silencing of KLF4 expression in differentiation media induced smooth muscle like morphology by day 5 in culture and increased overtime with expression of SMC markers in hSMSPCs. Implantation of silastic tubes into the rat peritoneal cavity induces formation of a tissue capsule structure which may be used as vascular grafts. Rat SMSPCs integrate into, strengthen and enhance the SMC component of such tubular capsules. These data demonstrate that KLF4 directly represses myocardin gene expression in hSMSPCs, which when differentiated, provide a potential source of SMCs in the development of autologous vascular grafts in regenerative medicine.

Abbreviations

ACTA2	Alpha-actin-2
ALK	Activin-like kinases
ANF	Atrial natriuretic factor
APC	Anaphase promoting complex
ATP	Adenosine triphosphate
BES	*N, N*-bis [2-hydroxyethyl]-2-aminoethanesulfonic acid
BMMSC	Bone Marrow derived Mesenchymal Stem Cell
BMP	Bone morphogenetic protein
CABG	Coronary artery bypass grafting
CAP	cAMP response element binding protein
cdk	cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
CHIP	Carboxyl terminus of hsp70-interacting protein) ligase
ChIP	Chromatin immunoprecipitation
CNN	Calponin
CoSMC	Coronary smooth muscle cells
CVD	Cardiovascular disease
CX3CR1	Chemokine (C-X3-C motif) receptor 1
CYP1A1	Cytochrome p450 1A1
DAPI	4',6-diamidino-2-phenylindole
dKO	Double knockout
DMEM	Dulbecco’s modified media
dpc	day post coitus
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extra cellular matrix
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGM	Endothelial Cell Growth Media
EGTA	Ethylene glycol tetraacetic acid
EKLF	Erythroid Krüppel Factor
EL	Endothelial like
ePTFE	Expanded Polytetrafluoroethylene
ERK	Extracellular-signal-regulated kinases
ESC	Embryonic stem cell
Ets	E-twenty six
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factors
Flk	Fetal Liver Kinase
Flt-1	fms-related tyrosine kinase 1
FOXO 4	Forkhead transcription factor 4

GFP	Green fluorescent protein
GKLF	Gut-enriched Krüppel-like factor
HASMC	Human Aortic Smooth Muscle Cells
HAT	Histone acetyltransferase
hCASMC	Human coronary smooth muscle cells
HDAC	Histone deacetylase
HeK 293T	Human Embryonic Kidney 293 cell variant expressing SV40 Large T antigen.
HERP	HES-related repressor protein
HLH	Helix-loop-helix
HMG2L1	High mobility group 2 like 1 protein
HMT	Histone methyltransferase
HOP	Homeodomain – only protein
hPGK	human phosphoglycerate kinase eukaryotic promoter
HPP-SOC	High proliferation potential SMC outgrowth cell
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cells
hSMSPC	Human Smooth Muscle Stem Progenitor Cell
HUVEC	Human Umbilical Vein Endothelial Cells
IAP	Intestinal Alkaline Phosphatase
Id	Inhibitor of DNA-binding/differentiation
Isl-1	LIM-homeobox transcription factor positive
JNK	Jun amino-terminal kinases
KLF	Krüppel-like factor
LKLF	Lung Krüppel like factor
MAPC	Multipotent adult progenitor cells
MAPK	Mitogen-activated protein kinases
MCIP	Multipotent *Isl*1+ cardiovascular progenitor
MEF	Myocyte enhancer factor
MEK	MAPKK, kinase of mitogen-activated protein kinase
MKL	Megakaryoblastic leukemia
MLC-2V	Myosin light chain
MLCK	Myosin light-chain kinase
MMP	Matrix metalloproteinase
Monc	Mouse neural crest cell line
MRF	Myogenic regulatory factors
MRTF	Myocardin related transcription factor
MSC	Mesenchymal stem cell
Mv1Lu	Mink lung epithelial cells
Myf	Myogenic factor
MYH11	Myosin heavy chain 11
NLS	Nuclear localisation signals
NTD	N-terminal domain
Oct-04	Octamer-binding transcription factor 4
OCT	Optimal cutting temperature

PBMNC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDA	Patent ductus arteriosus
PDGF	Platelet derived growth factor
PECAM	Platelet endothelial cell adhesion molecule
PMSF	Phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride
PRISM	PR domain in smooth muscle
qRT-PCR	Quantitative reverse transcriptase *PCR*
RA	Retinoic acid
RBM	RNA-binding motif protein
RhoA	Ras homolog gene family, member A
RLU	Relative luminescence units
rSMC	Rat Smooth Muscle Cells
rSMSPC eGFP	Rat Smooth Muscle Stem Progenitor Cell expressing eGFP
RT	Room temperature
Runx2	Runt related transcription factor 2
SAP	SAF-A/B, Acinus, PIAS
SBE	Smad binding element
Sca	Stem cell antigen
SMC	Smooth muscle cell
SME	Smooth muscle cell elements
SmGM	Smooth Muscle Growth Medium
SML	Smooth muscle like
SMMHC	Smooth muscle myosin heavy chain
SOC	Smooth muscle outgrowth cell
SOX-2	Sex determining region Y)-box 2
SRE	Serum response element
SRF	Serum response factor
SSEA-1	Stage-specific embryonic antigen 1
TAD	Transactivation domain
TCE	Transforming growth factor control element
TCF	Ternary complex factor
TDG	Thymine DNA glycosylate
TGF	Transforming growth factor
TSS	translation start site
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
WinMDI	Windows Multiple Document **I**nterface for flow cytometry

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1. Review of literature

1.1. Introduction

Cardiovascular disease (CVD) remains a leading cause of death in the Western world as well as in developing countries [1, 2]. Atherosclerosis, a major component of CVD, is caused by the accumulation of fatty materials, inflammatory macrophages and smooth muscle cells [3]. Vascular smooth muscle cells (VSMCs) line the walls of blood vessels and are spindle shaped cells range from 5-10μm in diameter which maintain the shape and tone of blood vessels. VSMCs are surrounded by basement membrane sheets of extra cellular matrix (ECM). ECM acts as scaffold for cell adhesion and plays a major role in cell differentiation, cell migration and cell proliferation. The major components of basement membrane are laminin, collagen IV and heparan sulphate proteoglycan perlecan [4]. Collagen IV maintains structural stability of ECM and heparan sulphate proteoglycan bridges laminin and collagen IV and also interacts with soluble growth factors [5].

1.2. Role of smooth muscle cells in Atherosclerosis

The arterial vessel wall is made of three layers, tunica intima (inner layer), tunica media (middle layer) and tunica adventitia (outer layer). The intimal layer consists of endothelial cells that line the lumen. The middle layer comprises smooth muscle cell, which controls vascular constriction and vasodilation whereas the outer layer includes connective tissue and its function is to attach the vessels to the neighbouring tissue [6].

Atherosclerosis (from Greek origin *athere* –meaning gruel, or wax and *sclerosis* - hardening) of arteries is the most common cause for heart attacks and stroke. The major factor responsible for this pathological process is apo-B containing lipoprotein retention and subsequent recruitment of smooth muscle cells and inflammatory macrophages and T-lymphocytes to the arterial wall [7, 8]. SMC accumulates in the intimal region, secretes extracellular matrix including collagen, elastin and proteoglycans leads to fibrous tissue formation, which is the characteristic component of the developing lesion [7]. Plaque rupture of the thin fibrous cap mediates plaque instability and perpetuates thrombosis which results in complete occlusion of artery leading to myocardial infarction [9-11]**.** SMC accumulation in the intimal region heals the ruptured plaque and also leads to intimal hyperplasia. This healing process narrows the artery and mediates arterial stenosis [12]. The developmental stages of atherosclerotic plaque are shown in Fig.1.1.

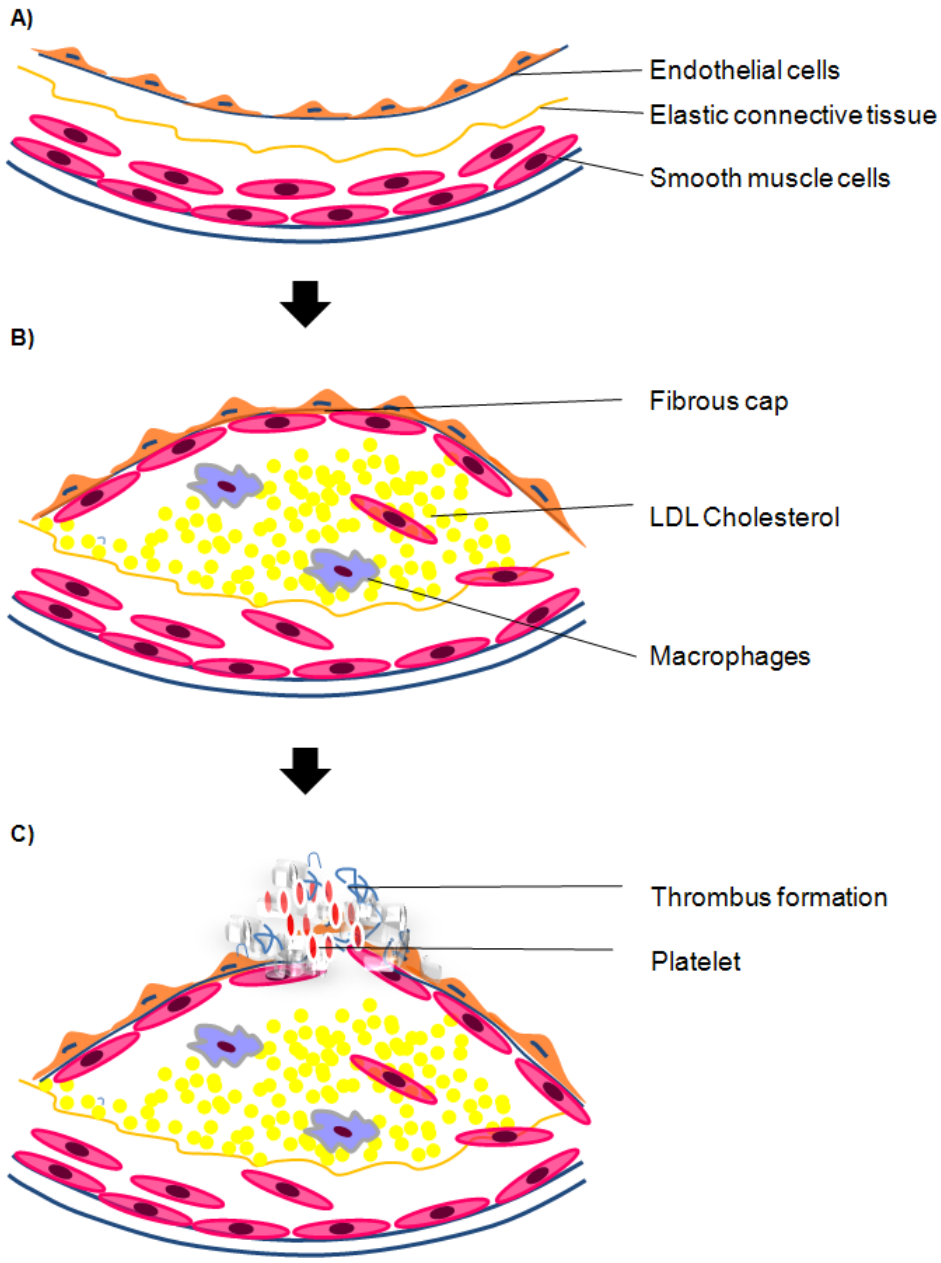


Figure 1-1. Developmental stages of atherosclerotic plaque.

A) Normal arterial wall showing inner layer with endothelial cells, elastic lamina and middle layer with smooth muscle cells. B) Artery with plaque development due to accumulation of LDL cholesterol recruitment of macrophages and smooth muscle cells results C) Artery with plaque rupture and thrombosis.

1.3. SMC in Vasculogenesis

Embryonic blood vessel formation involves vasculogenesis, which is the spontaneous organisation of blood vessel by endothelial cell differentiation from mesoderm. This endothelium monolayer recruits primitive SMC precursor cells from mesenchyme which differentiate into VSMCs and form a multilayered vessel wall

[13]. VSMCs in association with vascular endothelium produce extracellular matrix within the vessel wall and form mature blood vessels [14]. In normal arteries, the medial layer is composed of layers of contractile smooth muscle cells. During development, the smooth muscle cells are fibroblast like with larger endoplasmic reticulum, prominent golgi complex and lesser myofilament [15]. When the vessel matures, smooth muscle cells secrete more extracellular components [16] results in decrease in organelles size and increase in myofilament of the cell [15]. These studies suggest that SMC plays a major role in vasculogenesis as well as in development of vascular diseases.

1.4. Smooth muscle markers

Mature medial contractile SMCs express a range of unique smooth muscle lineage specific proteins including SM α actin [17], SM22α [18], SM1 and SM2 myosin [16], calponin, and h-caldesmon [16]. However, in response to various environmental cues such as vascular injury, cytokines and extracellular matrix, the differentiated SMC switch to a highly proliferative phenotype [19] characterised by a drastic decrease in expression of smooth muscle markers such as SM α actin [17, 20], SM22α [18], SM1 and SM2 myosin [18], calponin, and h-caldesmon [21, 22] (Fig.1.2), suggesting that identification of these multifunctional SMCs will be possible only through a clear understanding of the transcriptional regulation of smooth muscle marker genes.

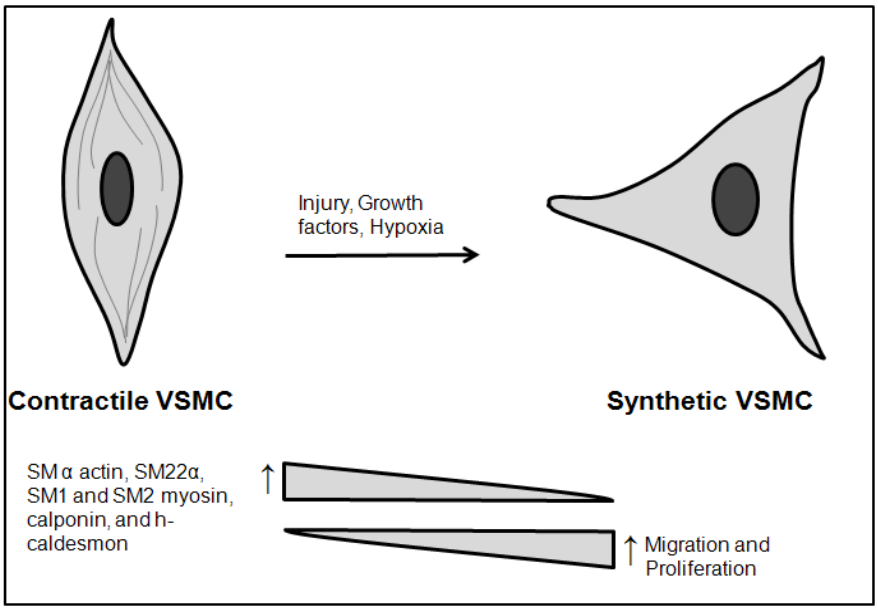


Figure 1-2. Phenotypic switching of vascular smooth muscle cells between contractile and synthetic state.

1.4.1. SM α actin

Actin is the most abundant cellular protein which is involved in cytoskeletal organisation and muscular contraction in the eukaryotic cell [23]. Actin is highly conserved across species, exists as six different isoforms [24] coded by separate genes and grouped into three classes α, β and γ based on their isoelectric point [25]. Alpha actins (α-skeletal, α-aortic smooth, α-cardiac and γ2-enteric smooth) are specific to muscle whereas beta and gamma isoforms (β- and γ1 –cytoplasmic) are prominent in non muscle. Vascular smooth muscle cells predominantly express alpha type SM α actin [25] and vimentin whereas other smooth muscle cell types in digestive, respiratory and urinary tracts express gamma type SM α actin and desmin [26]. SM α actin is 43 kDa protein encoded by ACTA2 gene and its main function is to generate force in VSMCs during contraction [27, 28]. SM α actin is the early marker expressed in differentiated SMCs which arise from mesenchyme during vasculogenesis [29]. In early vasculature, β-actin content is higher than α actin. As vessels mature, the SM α actin content increases significantly and occupies 70% of actin content in VSMCs [30]. SM α actin has also shown to be expressed in pericytes [31], myofibroblasts [32] and tumours [33]. In spite of their abundance and early occurrence during differentiation, SM α actin is not an exclusive marker for vascular smooth muscle cell lineage due to their expression in other cell types.

1.4.2. Smooth muscle myosin heavy chain (SMMHC)

Myosins are motor proteins involved in muscular contraction. Myosins are classified into 20 different classes [34] based on phylogenetic analysis of a conserved domain [35]. Different myosins show variation in their tail domain whereas head domain sequences are highly conserved among them. The class II myosins in human have 15 genes, six skeletal myosin heavy chains [36], two cardiac myosin heavy chains, one smooth muscle myosin heavy chain and two non-muscle myosin II heavy, respectively [37]. Myosin is a hexamer protein made of two myosin heavy chains and two set of myosin light chains [38].

Smooth muscle myosin heavy chain (200-204 kDa protein) is one of the exclusive markers for vascular smooth muscle cells [39]. In situ hybridization studies in mice demonstrated that the expression of SMMHC was confined to smooth muscle tissues and was first detected in the developing aorta at 10.5 postcoitum [39, 40]. Furthermore, the expression of MHC isoforms was not detected in cells other than smooth muscle lineage [39], suggesting that SMMHC can be used as an exclusive markers for SMCs

Previous studies have identified many isoforms of smooth muscle myosin heavy chain generated *via* alternate splicing of single gene (MYH11). Alternate splicing in the 3' region produces SM2 (200kDa) and SM1 (204 kDa) isoforms [40, 41] whereas alternate splicing in the 5' region coupled with insertion of *exon 5b* (seven - amino acid insert) at the 25-50 kDa junction of the myosin produces SMB isoform and without *exon 5b* insertion produces SMA isoform [42, 43]. The use of both splicing sites produces four different isoforms SM1A, SM1B, SM2A and SM2B [44]. The presence of a seven amino acid insert sequence in SM1B is associated with higher actin activated ATPase activity, suggesting that presence of SM1B may contribute to varying functional properties of SMCs in different tissues [45].

A number of studies demonstrate that the expressions of SMMHC isoforms are differentially regulated during development of SMCs in a tissue specific manner [46]. The SMB isoform showed a predominant expression in all stages of bladder development. The expression of the SMB isoform was detected at 1 week in neonatal rat aorta but the transcript level decreased to an almost undetectable level in the adult aorta [46]. VSMC in aorta express both SM1 and SM2 isoforms [40, 47] which are developmentally regulated; the SM1 isoform is expressed in VSMC from the late fetal stage throughout adult, whereas the SM2 isoform expression appears only in fully differentiated SMCs after birth [21, 48]. VSMCs isolated from aorta continue to express only the SM1 isoform but the expression of SM2 isoform is downregulated in culture [49, 50]. During atherosclerotic lesion development, phenotypic modulation of VSMCs was accompanied by reduction in SM2 isoform with marked increase in SMB isoform [21]. Taken together, these studies suggest that the expression of smooth muscle myosin heavy chain (SMMHC) is developmentally regulated and smooth muscle myosin heavy chain (SMMHC) isoforms can be used as exclusive markers to study VSMC differentiation as well as for atherosclerosis [51].

1.4.3. Calponin

Calponin (CNN) is a troponin –T like myofibrillar thin filament 28-34 kDa protein and was originally isolated from chicken gizzard smooth muscle and bovine aorta [52, 53]. Calponin is involved in stabilization of cytoskeletal actin [54] and regulation of smooth muscle contraction [55]. Three isoforms of calponin, named h1, h2 and acidic calponin have been identified in smooth muscle cells [56] and are found to be transcribed from three different genes [57]. Calponin family protein consists of three domains, the N-terminal calponin domain, the middle actin binding domain and the C-terminal domain. Calponin isoforms are distinguished from each

other by their unique C-terminal tails [58]. Based on the isoelectric points (pls), calponin isoforms are categorised as basic or h1-calponin (CNN1) with pI = 8.5–9.2, acidic calponin (CNN3) with pI of 5.5 to 5.8 and neutral or h2 calponin (CNN2) with pI = 7.2–7.6, respectively. [59, 60]. Two dimensional gel electrophoresis analyses has identified a spliced variant of α calponin named β calponin (28 kDa protein) and its expression was predominantly detected in urogenital tract smooth muscle cells [61]. Neutral (h2 calponin) [57] and acidic calponin (h3 calponin) [56] are encoded by two different genes and are expressed in lower levels in smooth muscle and non muscle cells. Neutral calponin (CNN2) has been found to be expressed in heart [62] and in remodelling smooth muscle cells [60]. However, acidic calponin (CNN3) was expressed predominantly in rat brain [56] and shown to facilitate wound healing activity by extracellular-signal-regulated kinases 1/2 (ERK1\2) activation and enhance cell motility in fibroblast [63].

Basic calponin (CNN1) is the most abundant form present in differentiated smooth muscle cells [61] and modulates contraction of smooth muscle cells [55]. In contrast, expression of h2 calponin is detected in non muscle cells such as heart [62], endothelial cells and fibroblast [64]. Of note, h2 calponin has also been detected at elevated levels in remodelling and growing SMCs in culture [60].

Calponin binds to F-actin and regulates actin myosin interaction by blocking the activation of myosin ATPase in calcium dependent manner. An increase in intracellular calcium levels activates the calcium calmodulin complex formation. This complex binds to calponin and releases the actin molecule to interact with phosphorylated myosin resulting smooth muscle contraction [55]. Calponin phosphorylation by calcium calmodulin kinase II also disassociates actin and induces smooth muscle contraction in calcium independent manner [55]. The basic h1-calponin (CNN1) is predominantly smooth muscle specific and its expression is strongly downregulated in dedifferentiated SMCs and passaged VSMCs in culture [61, 65].

Gimona [66] and co workers investigated changes in calponin expression in smooth muscle cells from chicken gizzard and non muscle tissues during development based on monoclonal antibody for calponin. Results revealed that SMCs from chicken gizzard expressed increased differentiation linked calponin expression. In contrast, tissues from non muscles such as chicken skeletal muscles, kidney, spleen, liver and cardiac muscles showed no calponin expression [66], suggesting that calponin expression may be used as index to measure smooth muscle differentiation.

Expression of h1-calponin is developmentally regulated in VSMCs. In developing dorsal aorta, cardiac outflow and tubular heart calponin was detected in E9.5 murine embryos [67]. Expression of h1-calponin increased drastically in vascular smooth muscle cells whereas expression of h1-calponin progressively decreased to undetectable levels in heart [67], suggesting that the expression of h1-calponin may be used as an indicator for contractile SMC phenotype.

In summary, these studies indicate that smooth muscle markers and their isoforms may be used for assessing the various stages of differentiation/proliferation of the smooth muscle cells.

1.5. VSMC and phenotypic modulation

VSMCs present in the arterial media are maintained at the G0/G1 resting phase of the cell cycle and exhibit high level of contractile smooth muscle marker genes[68]. However, in response to vascular injury and subsequent release of growth factors, VSMCs attain a highly proliferative synthetic phenotype [69]. This process which changes the contractile to a synthetic state is called phenotypic modulation or SMC plasticity [70, 71] (Fig.1.2).

Smooth muscle cells are the major extracellular matrix generators in blood vessels [14]. During blood vessel development, the immature SMCs are in a dynamic state with high proliferation and migration potential [72]. As vessels mature, the SMCs becomes quiescent [73] and fully differentiated with characterised expression of contractile phenotypic markers. SMC phenotype can switch between the dynamic and quiescent state upon local environmental cues. Unlike the cardiac and skeletal muscle, the SMC are not terminally differentiated. Phenotypic modulation of SMCs occurs during pathological conditions and helps SMCs to attain the highly dynamic state to exhibit multiple states of growth and differentiation. The sequence of events that takes place during pathological intimal thickening resembles the neonatal intimal thickening during development which explains that intrinsic migration potential of SMC may not be age related [74] but its migratory potential is controlled by local environment. The decision to undergo phenotypic modulation is aided by different growth factors produced in the cellular environment. Therefore a key for understanding the SMC differentiation and phenotypic modulation is to identify transcription factors that regulate smooth muscle specific genes that results in the distinct phenotype of the VSMC [22, 75].

The expression of skeletal and cardiac muscle specific genes is developmentally regulated [76] and many master control genes have been identified

which alone control the skeletal muscle lineage [75]. Skeletal and cardiac muscles express overlapping sets of muscle specific genes. Skeletal muscle specific transcription is activated by MyoD family of regulatory factors whereas cardiomyocytes have unique cell type specific helix-loop-helix (HLH) protein for activation of cardiac muscle specific gene activation [76]. MyoD family proteins belong to the basic-helix-loop-helix (bHLH) transcription factor. They include MyoD, myogenic factor 5 (Myf5), myogenin, and myogenic regulatory factors 4 (MRF4) and they share 80% homology within a segment of 70 amino acids that contain a basic region and a helix-loop-helix conformation [77]. HLH motif dimerization brings the basic region together and forms the DNA binding domain which recognizes the dyad symmetrical DNA sequences CANNTG (N is nucleotide) called E boxes [78], found in 5' flanking regions of skeletal muscle specific genes and activate transcription in association with ubiquitous bHLH proteins referred as E proteins [79, 80]. Over expression of the MyoD family genes in non muscle cells can activate a skeletal muscle differentiation is program in non muscle cells [80]. Specificity of MyoD in skeletal muscle specific gene transcription referred to a master regulatory gene. Inhibitor of DNA-binding/differentiation (Id) protein, an inhibitor for differentiation also belongs to the HLH family of protein. Id proteins lack the DNA binding domain but they can negatively regulate the function of MyoD protein through the formation of non functional heterodimer. Id expression is high during proliferation of skeletal muscle and down regulated during differentiation [81]. Thus, skeletal muscle development and differentiation is well defined and its transcription machinery is controlled by a master regulatory gene.

In contrast, SMC transcription is not controlled by a master transcription factor. Moreover, in response to various cytokine stimuli SMC undergoes dedifferentiation from its contractile phenotype to synthetic phenotype which is the underlying mechanism for many vascular diseases. This is described as phenotypic modulation of SMC which is accompanied by downregulation of differentiation markers SM α actin, SMMHC and SM22α. The key challenge in the SMC field is to determine transcriptional regulation of SMC under various physiological and pathological conditions. Extensive research has been carried out in the last decade in identifying transcriptional regulators of SMC.

1.6. Transcriptional regulation of smooth muscle cells

1.6.1. CArG Element

Comparative promoter analysis of the human, mouse and chicken cardiac α actin gene showed the presence of common motif coined as the CCArGG box (CC (A/G-rich) GG which is conserved in evolution [82]. From here, CCArGG box abbreviated as CArG box. CArG boxes are found in muscle restricted genes and growth related genes which are controlled by SRF. The CArG box was identified as a core sequence of the serum response element (SRE) in the c-fos gene [83] with a single CArG box in its promoter region whereas most smooth muscle markers have at least two CArG boxes located in their promoter and first intronic regions [84-87]. CArG boxes are located in the promoter region of smooth muscle (SM α actin, SM22α, SMMHC, telokin, calponin etc), cardiac (cardiac α actin, βMHC and dystrophin [82, 88-90] and skeletal muscle α actin genes [91]. They are also located in many immediate early genes including erg1, erg2 and β actin [92].

The SM α actin promoter has three CArG elements CArG A, CArG B and intronic CArG located at -62, -112 and +1001 bp, respectively. The spacing and phasing of the SM α actin CArG element are important for its promoter function. The CArG A and CArG B are located in close proximity with 40 bp distance between them on the same side of the DNA helix. Changes in the distance between CArG element and phase of CArG element may abrogate the SM α actin promoter activity, suggesting that spacing between the paired CArG elements are critical for SRF and its cofactor binding/interaction in SMC promoter transactivation [93].

SMC subtypes use distinct transcriptional control for activation of smooth muscle specific genes. SMMHC gene expression is highly restricted to SMC and expressed throughout development and maturation [39]. SMMHC has three CArG boxes in its promoter region and is used distinctly in SMC subtypes. Transgenic mice harboring a conserved SMMHC promoter reporter construct (–4200 to +11600 region of SMMHC gene) showed that SMC specific expression of SMMHC gene requires 4.2 Kb of the 5' flanking sequence, the entire first exon and 11.5 Kb of the first intronic sequence in all subtypes of SMCs. CArG2 box located at -1231bp and the intronic CArG element is an absolute necessity for SMMHC expression in large blood vessel whereas it has minor role in controlling expression of SMCs in gastrointestinal and urinary tract SMCs. Thus, SMMHC gene expression is differentially regulated by the presence of multiple CArG elements within its promoter region in SMC subtypes. [86]. Similarly, transcription of SM22α in cardiac

and smooth muscle cells is dependent on presence of two CArG boxes in its promoter region. SM α actin gene has three CArG elements located within -2.6/+2.8 Kb of the promoter enhancer region. [94]. Taken together, location and number of CArG box present in the promoter region determines the tissue specific expression in SMC subtypes.

Smooth muscle marker CArG elements are degenerate compared with the conserved consensus CArG element of c-fos promoter [95, 96]. The SM marker CArG element has G or C substitution within their A/T cores and highly conserved in evolution [95, 97] and plays a major role in SMC selective regulation during development and vascular injury. The G/C substitution in CArG elements mediate reduced SRF binding in SMC as compared to c-fos CArG element with high SRF binding affinity [95, 96, 98]. Smooth muscle genes showed a significant transcriptional repression seven days after vascular injury and their expression level returned to nearly equal to control level by 14 after vascular injury [98, 99]. Substitution of consensus CArG elements from c-fos to both 5' CArG elements of SM α actin promoter showed no difference in SMC specific expression whereas the transgenic mice showed an attenuated SM α actin expression level after vascular injury. In addition, the SRF expression level was increased but expression of myocardin was decreased within three days and returned to nearly equal to control level by seven days after carotid injury [98]. Further, myocardin increased SRF interaction with CArG element of SM α actin promoter but not of c-fos promoter region, suggesting that G/C substitution within 5'CArG element (CArG degeneracy) within the SMC promoters and decreased myocardin expression level plays a critical role in phenotypic switching of SMC in response to vascular injury [98].

1.6.2. Serum response factor (SRF)

SRF, a MADS (MCM1, Agamous, Deficiens, SRF) box transcription factor is expressed in a wide range of cell types and was first identified as SRE binding protein by its ability to activate c-fos gene upon serum stimulation [100]. SRF has four isoforms known as SRF-L, SRF-M, SRF-S and SRF-I generated through alternative splicing and its expression is regulated in a tissue specific manner. SRF-M and SRF-L are expressed at similar levels in differentiated smooth muscle and skeletal muscles but SRF-L is predominantly expressed in other tissues. SRF-S and SRF-I expression are exclusive to differentiated VSMC and embryo respectively [101]. SRF binds on CArG box [102] as homodimer [100] and recruits a variety of

transcriptional cofactors and activates transcription of most of the muscle specific gene expression [103], [104].

The c-fos CArG element has a higher SRF binding affinity compared to many SMC CArG elements. SRF binding affinity is dependent on the G/C nucleotides within the 5' CArG element. For example, the SM α actin CArG element contains a single G or C substitution in A/T rich region of CArG element found to be responsible for its low SRF binding affinity. SMC with abundant SRF enhances its binding affinity to degenerative CArG elements [95, 105]. SRF activity can be modulated through its prolonged DNA binding to CArG element [106]. Homeodomain protein MHox induced by angiotensin II mediates SRF binding to SM α actin CArG element [107]. Another, homeodomain protein Barx2b, found to enhance SRF binding to telokin CArG element [108]. In contrast, homeodomain – only protein (HOP) physically associates with SRF and inhibits the SRF to bind to CArG element [109, 110]. Thus, SRF protein: protein interaction can modulate SRF binding to CArG element.

SRF is a weak transcriptional activator but it's able to interact with over 60 cofactors and attain its strong transcriptional activator potential in a tissue specific manner. SRF directs the gene expression mainly through two signalling pathways [111, 112]. First, the classical pathway involves growth factor stimulation phosphorylation of SRF through mitogen-activated protein kinases **(**MAPKinase) pathway which results in growth related genes activation [113, 114] and the second is the Ras homolog gene family, member A (RhoA) pathway of actin dynamics [115], which involves nuclear translocation of myocardin related transcription factor A (MRTF-A) in response to F actin and activation of SRF dependent genes [116]. Furthermore, SRF dependent growth related genes encode for protein that activates cell cycle genes [117]. Thus SRF is an indirect controller of cell growth and the contractile apparatus. Presence of an Ets (E-twenty six) binding sequence (GGAA/T) adjacent to CArG element in most of the smooth muscle marker gene promoters and in growth related genes enables SRF to toggle between programs of differentiation and cellular growth. Stimulation of MAPKinase signalling cascade by epidermal growth factor (EGF) phosphorylates SRF [118] which interacts with ternary complex factor (TCF) and activates transcription of c-fos gene via SRE element. In addition to SRF, c-fos activation involves other transcription factor interaction with SRF and SRE [119]. ETS domain-containing protein, Elk-1 is one such factor which forms a ternary complex with SRF bound to SRE element and activates c-fos gene in response to protein kinase C dependent signalling [120]. Elk-

1 is member of the ternary complex factor (TCF) subfamily of the ETS domain transcription factor and is activated via phosphorylation of MAPK pathway [121]. Elk-1 contains conserved N terminal ETS domain with high sequence specific DNA binding potential [122, 123], the B–Box which directs protein: protein interaction with SRF [124] and the C terminal transactivation domain target for phosphorylation by MAPK pathway [125, 126]. The Elk-1 association with SRF is stabilized by additional binding of its ETS domain to ETS sequences. [121, 127]. MAPKK, kinase of mitogen-activated protein kinase 1/2 (MEK 1/2) dependent phosphorylation of ERK-1/2 phosphorylates Elk-1 and stimulates its interaction with SRF [128]. Phosphorylated Elk (Elk-p) then competes with myocardin for the SRF docking site which displaces myocardin and downregulates smooth muscle gene expression [127]. In addition, Elk-1 binding to Ets binding sequences adjacent to CArG box partially blocks myocardin association with SRF [129]. Thus, SRF modulates genes involved in growth and differentiation of smooth muscle cells through changing its interaction with Elk-1 and myocardin.

SRF plays a critical role in embryogenesis during mesoderm differentiation [130] and transcriptional regulation of cardiac muscle genes. SRF null embryos failed to develop beyond E6.0 and showed lethality with impaired mesoderm development. $SRF^{-/-}$ embryonic stem cells in mouse showed defects in muscle specific gene expression and in immediate early genes [131, 132]. Inactivation of SRF in cardiomyocytes attenuates the sarcomeric proteins necessary for its contractility [133]. In mouse, cardiac and smooth muscle specific deletion of SRF results in lethality. Skeletal muscle specific SRF knockout leads to perinatal lethality with severe skeletal muscle myopathy in mice [134]. Taken together, these data suggest that SRF is critical for regulation of cardiac, smooth and skeletal transcriptional regulation.

The structure and function of chromatin is predominantly through posttranslational modification of histone lysine residues methylation and acetylation by histone acetyltransferase (HAT) and histone methyltransferase (HMT) activity [135]. The acetylation process unwinds the chromatin whereas methylation can serve as a docking site for transcription factors on chromatin [136, 137]. This epigenetic mechanism is well documented in regulation of SMC-specific SRF binding to CArG elements. SMC specific SRF activation is also determined by an epigenetic control mechanism through posttranscriptional histone modification which includes methylation and acetylation of histone H3 and H4 residues. Myocardin utilizes smooth muscle specific epigenetic element (H3K4dMe) and controls SRF

binding to CArG box DNA. Presence of SMC restricted myocardin and H3K4Me mediates SRF to activate smooth muscle specific genes [138]. In contrast, upon vascular injury, KLF4 binds to the evolutionary conserved TCE elements adjacent to CArG box [139, 140] which recruits HDCA2 to deacetylate histone H4 [141] and repress myocardin/SRF activation of smooth muscle specific genes.

In summary, posttranscriptional modification, associated with positive and negative cofactors, binding affinity to CArG box, the presence of different isoforms explain how SRF can activate a wide range of genes involving exclusive events like growth and differentiation [93, 103].

1.6.3. Myocardin

The major breakthrough in understanding gene regulation in SMC was the identification of a novel and potent transcriptional cofactor; named myocardin using bioinformatics based screening of unknown cardiac specific genes. Myocardin belongs to SAP domain family of nuclear proteins and is highly expressed in developing cardiac and smooth muscle tissue [142]. In mouse embryos, myocardin expression was first detectable in the cardiac crescent at E7.75 stage whose expression is concurrent to other earliest cardiac specific marker, Nkx2.5 gene. Myocardin appears at E13.5 within smooth muscle cells lining the walls of the esophagus, aortic arch arteries and pulmonary outflow tract but myocardin expression was undetectable in skeletal muscle at any stage of embryonic development [142]. Myocardin is a highly potent cofactor for SRF and its expression is predominantly nuclear localised. Myocardin strongly transactivates cardiac specific promoters of SM22α, atrial natriuretic factor (ANF), myosin light chain (MLC-2V), and αMHC genes and smooth muscle specific promoters of SM α actin, SMMHC, calponin and telokin. [142]. SRF expression remains the same with phenotypic modulation from aortic media to cultured SMC whereas myocardin expression was attenuated in cultured SMC which shows a possible role of myocardin in modulation of smooth muscle transcription [143].

The human myocardin gene has five different splice variants (*Myocd_v1, Myocd_v2, Myocd_v3, Myocd_v4* and *Myocd_v5*) encoded by a single gene with 15 exons [144] located on chromosome 17p11.2 and shows 74% homology to mouse myocardin gene [145]. Myocardin has unusually long 3' UTR region (>4kb) and intron 1 exhibits high homology between species which suggests the presence of regulatory sequences in the control of gene expression**.** The presence of different splice variants differentiates the function of myocardin between cardiac and smooth

muscle cells. Splice variants *Myocd_v1* and *Myocd*_v2 were specific to cardiac lineage whereas *Myocd_v3* and *Myocd_v4* were specific to smooth muscle cells. Alternate splicing of *exon 2a* and *exon 10a* yields cardiac and smooth muscle specific splice variants. The cardiac specific myocardin variant *Myocd_v1* is the longest transcript coding for a protein with 983 amino acids whereas another cardiac specific variants *Myocd*_v2 lacks *exon 10a* (48 amino acid) with 935 amino acids. Both the cardiac variants have 79 amino acids extension for myocyte enhancer factor-2 (MEF2) binding site in its C-terminal region.

The smooth muscle specific myocardin also has two splice variants; both include *exon 2a* in their transcripts [146]. Inclusion of *exon 2a* introduces the stop codon and generates in a truncated 79 amino acid protein and the presence of start codon in *exon 4* encodes the most abundant smooth muscle specific myocardin *Myocd*_v3 with 904 amino acids. The rare smooth muscle specific variant *Myocd*_v4 (856 amino acids) has no *exon 10a* in its coding region. *Exon 10a* is rich in proline and serine residues and its role in signalling should be further investigated. Recently, *Myocd*_v5 (807 amino acid) variant has been identified in transactivation of SMC gamma actin promoter (*Acta2*) in association with Nkx3.1 homeodomain protein [144].

The N terminus of smooth muscle specific variants has two conserved RPEL (Arg-Pro-X-X-X-Glu-Leu) domains whereas cardiac variants have three RPEL domains. A seven residue sequence called B1 domain, which is between the basic region and glutamine rich Q domain [142, 147], mediates binding to SRF [148]. The SAP domain with 35-amino acids controls nuclear organisation, chromatin dynamics and apoptosis [149]. A Leucine zipper domain follows the SAP domain which is involved in homo-heterodimerization of myocardin family members in RhoA/actin dependent signalling process [116, 150] (Fig.1.3). The C–terminus of myocardin has strong transactivation (TAD) domain [151]. Myocardin and MRTFs (MRTF-A and MRTF–B) has similar protein structure with 35% overall protein homology. The basic, Q and SAP domain of MRTFs possess high sequence homology (60%) whereas TAD (28%) shows very low level sequence identity to myocardin [152].

SRF has been found to interact with other transcription factors like Nkx2.5 [153] and GATA4 [154] to activate cardiac specific genes. However, the protein-protein interaction between SRF and myocardin differs from SRF other factors interaction with SRF dependent transcription Moreover, SRF was unable to form a

stable ternary complex either with Nkx2.5 or GATA4 which results in relatively modest transcription activation as compared to myocardin [142].

Myocardin interacts with another MADS box protein other than SRF. MEF2 family transcription factors controls cardiomyocytes differentiation [155]. Expression of MEF2 single gene is sufficient to commit to cardiac lineage in drosophila [156, 157]. MEF2 contains MADS domain homology to SRF and binding site CTA (A/T) 4TAG similar to CArG box [142]. MEF2 binds to 79 amino acids NTD (N-terminal domain) of cardiac specific myocardin and activates cardiac specific genes [146] (Fig.1.3). Myocardin uses different domains to interact with two MADS box proteins (SRF and MEF2) to activate cardiac and smooth muscle lineage genes.

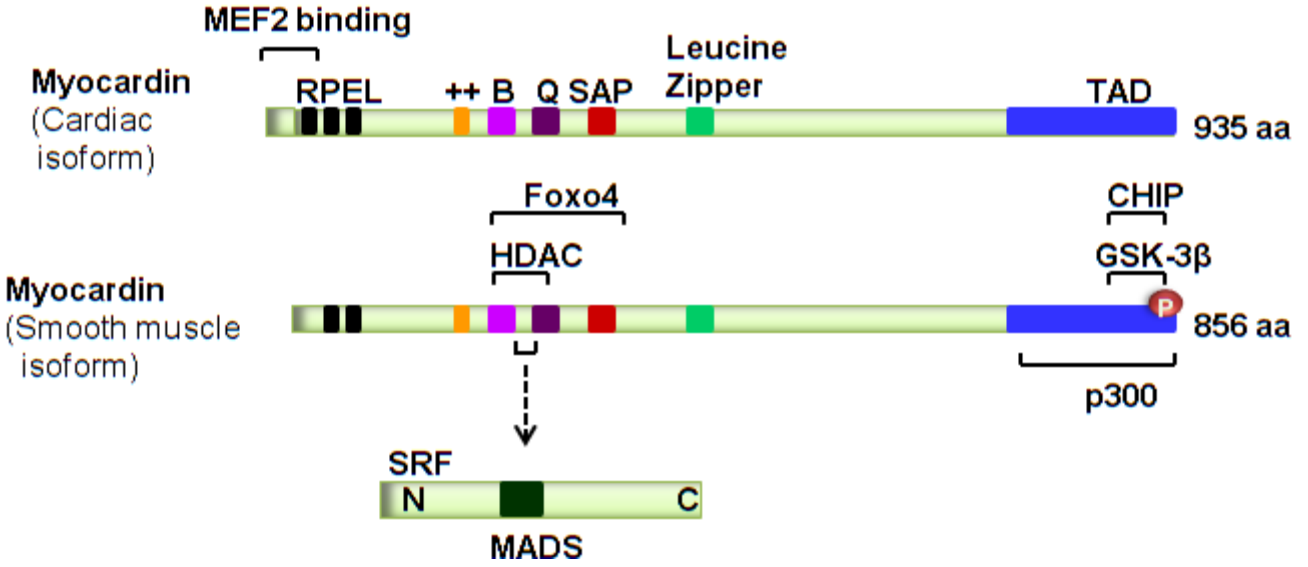


Figure 1- 3. The domain structures of human myocardin protein.

Cardiac specific isoform has 79 amino acids extension for myocyte enhancer factor-2 (MEF2) binding site in its C-terminal region. The basic B (++) and Glutamine (Q) rich domain facilitates myocardin binding to SRF. The Leucine zipper domain participated in homo–hetero dimerization of myocardin with other MRTFs. Myocardin has TAD, a powerful transactivation domain. The p300 binds to TAD domain and enhance the transactivation of myocardin whereas repressor proteins bind to the myocardin protein and repress its activity. Foxo4 bind to basic, Glutamine rich (B) and Sap domain and inhibits myocardin activity. HDAC binds to basic, Glutamine rich (B) and induces chromatin remodelling.GSK-3β (Glycogen synthase kinase 3β) phosphorylates the TAD domain of the myocardin protein. CHIP (Carboxyl terminus of hsp70-interacting protein) ligase binds to the C-terminal of myocardin and the phosphorylation of myocardin by GSK-3β enhances CHIP mediated ubiquitination and proteasome degradation of myocardin protein.

Myocardin deficient mice created by homologous recombination die by embryonic day (E) 10.5 due to delayed development and pericardial effusion [158]. The mutant homologous mouse has no evidence for VSMC differentiation but has normal cardiac development which indicates that myocardin is necessary for VSMC differentiation. Cardiomyocyte-restricted null mutation in mice developed cardiomyopathy accompanied by defects in cardiomyocytes structural organisation and apoptosis [159]. In xenopus, expression of dominant negative myocardin mutant in the dorsal vegetal blastomere shows defects in cardiac lineage [142] suggesting that myocardin is used differentially by VSMC and cardiomyocytes [160], myocardin is an absolute requirement for VSMC differentiation whereas it is necessary for maintenance of differentiated cardiomyocytes survival.

In most of the muscle as well as growth related genes, the CArG box resides within 2-3 kb of the translation start site (TSS) and relatively close to the RNA polymerase II holoenzyme. SRF binds to the CArG box and creates an acute bend on the genomic DNA which enables SRF to establish multi-protein complexes with other transcription factors [106, 161]. Such structure on the DNA across CArG box helps myocardin to interact with SRF through its Q region and transactivates smooth muscle gene transcription [106, 161]. Myocardin transactivates the SM calponin promoter with three conserved intronic CArG [162] elements resulting in 10 fold increase in SMC and mutation in CArG elements abolished myocardin induced transactivation showing the requirement of functionally active CArG bound SRF for its promoter activation [143]. These results suggest that activation of SM marker by myocardin is SRF- CArG dependent manner.

Myocardin, the promyogenic activator in association with p300, histone acetyltransferase and histone deacetylase 5 (HDAC5), a class II HDAC, modulates the SMC transcription. The p300 binds to the transactivation domain from 713 to 935 residues whereas the HDAC5 binds to 268 to 328 residues near to the N terminus of myocardin [163]. Chromatin immunoprecipitation assay in myocardin expressed 10T1/2 cells showed the evidence for histone H3 acetylation of CArG box located in SM α actin and SM22α promoter regions and the cotransfection of myocardin and p300 resulted in activation of 4X CArG luciferase reporters linked to SM22α promoter in COS cells [163]. In addition p300 enhanced the transcriptional activity of UAS luciferase reporter by GAL-4 DNA binding domain fused myocardin (GAL-4-Myo) and HDAC5, strongly repressed the p300 induced GAL-4-myocardin transcriptional activity. Chromatin immunoprecipitation (ChIP) of both p300 and

HDAC5 in the presence of myocardin indicated that they interact with separate domains, these results suggests that myocardin in association with the p300 (acetylation) and HDAC5 (deacetylation) can modulate the CArG-SRF dependent SMC transcription [163].

It is interesting to note that the non SMC L6 cell line, expresses neither myocardin nor SMC markers. Ectopic expression of myocardin induced the expression of calponin, SM α actin and ANF as measured by RT-PCR, suggesting that myocardin can activate the smooth muscle differentiation program in non SMC [143].

1.6.4. Krüppel like transcription factor 4 (KLF4)

Multiple repressor proteins interact with myocardin and suppress myocardin induced transactivation [164] either by blocking myocardin SRF interaction [165] or by mediating posttranslational modification of myocardin and further degradation by proteasome [166]. KLF4 is one of the most studied repressor proteins associated with suppression of myocardin induced SMC transcription [167].

In general, the structure of eukaryotic transcription factors consists of DNA binding and transactivation domains, the later having binding sites for coactivators. Transcription factors are classified into super classes based on the DNA binding domains into 1) basic domain 2) zinc coordinating DNA binding domain 3) helix-turn helix and 4) beta scaffolding factors with minor groove contacts [168, 169]. KLFs are a subfamily belonging to class Cys2His2 zinc finger transcription factor and they are highly conserved among mammals [170]. The KLFs are named after the drosophila segmentation gene Krüppel (Cripple in German), with which their DNA binding domain show homology. [171]. KLFs contain a highly conserved characteristic C-terminal binding domains with three C2H2 zinc fingers, each chelating a zinc ion in the middle, co-ordinated by two cysteine and two histidine residues [172]. KLFs have a highly conserved H/C link, a seven sequence residue TGEKP(Y/F)X between the zinc fingers [173]. The DNA binding specificity is directed by three critical residues within each zinc finger [171]. In contrast, the N-terminal transactivation domains of KLF are highly divergent, vary significantly and allow them to bind with various coactivators such as cAMP response element binding protein (CBP), p300, and p300/CBP- associated factor (P/CAF) and corepressors like histone deacetylase (HDAC) resulting in its functional diversity and specificity [170].

KLF4 (GKLF- Gut-Krüppel like Factor) is a zinc finger transcription factor belonging to the Krüppel like factor family transcription factors. KLF4 is expressed

in variety of tissues and plays a major role in proliferation, differentiation, development and apoptosis [174]. KLF4 was first identified and characterised independently by two groups, named as GKLF [175] and EZF [176] (renamed as KLF4) due to its high expression in epithelial lining of intestine and skin, respectively. KLF4 is also expressed in lung, testis, thymus, cornea, cardiac myocytes and lymphocytes. [174]. The human KLF4 (54 kDa) protein is encoded by KLF4 gene locus located on 9q31 [175]. KLF4 null mouse die shortly after birth due to dehydration and defects in barrier function of the skin. [177]. In addition, KLF4 homologous mice showed a loss of goblet cells from the colon [178], suggesting that KLF4 plays a critical role in epithelial and colonic cell differentiation.

1.6.4.1. Structure of KLF4

KLF4 protein has three distinct domains such as N-terminal activator domain, a central repressor domain and the C-terminal DNA binding domain [176, 179]. KLF4 has two potent nuclear localisation signals (NLS), first NLSs is located in the 5' basic region (immediately amino terminal to the zinc fingers) which includes four arginine and lysine within a hexapeptide, (PKRGRR) between 385-390 amino acid residues. The second NLS resides within the zinc fingers, since the one and half amino terminal zinc finger itself confers a nuclear localisation signal in KLF4 [180]. In addition, KLF4 has a PEST (proline (P),glutamine (G),serine (S) and threonine (T)) sequence acting as a signal peptide for ubiquitin-mediated proteasomal degradation associated with the intracellular short half lives ($t_{1/2}$ ~120 minutes) of proteins. KLF4 contains a PEST domain (113 to 132 aa) located between the transcriptional activation and inhibitory domain (Fig 1.4) with high PEST score of 5.8 and relatively short half life [175, 181].

The zinc finger region of KLF4 shares a high degree of sequence homology with LKLF (lung Krüppel like factor) at 92%, EKLF (Erythroid Krüppel like Factor) at 84% and BTEB2 (KLF5) at 82% respectively. KLF4 also has 52% of sequence homology to Sp1 transcription factor [175]. Furthermore, KLF4 bind to GC-rich (5'–GGG GCG GGG-3') sites on DNA which is similar to KLF5 and specificity protein (SP1) binding site. However, the amino acid sequence outside the zinc finger domain have no homology with other KLFs, suggesting that the KLFs can recognise same binding sequences on DNA but exert different functions due to the coregulator recruitment coupled to their divergent N terminal transactivation domain [175].

1.6.4.2. Mechanism of KLF4 activation and repression

The N terminal region of KLF4 is rich in proline, serine, threonine and acidic amino acid residues associated with activation or repression of transcription [182]. Presence of acidic residues in the N-terminal region enhances binding of p300/CBP coactivators. KLF4 binds to the GC rich region within the promoter of target genes and recruits the coactivators like p300/CBP proteins which have catalytic histone acetyltransferase (HAT) activity. The p300 protein directly acetylates KLF4 at Lys 225 and Lys 229 amino acid residues and neighbouring H4 histones on the promoter resulting in transcriptional activation of target genes [183]. Furthermore, mutation analysis of two lysine to arginine residues attenuates the transactivation potential of KLF4 indicating that acetylation of KLF4 itself is important for its transcriptional activity. KLF4 binds to IAP (Intestinal Alkaline Phosphatase) promoter and recruits p300, transcriptional coactivator which mediate histone H4 acetylation and transactivation of the target gene [183]. However, KLF4 can function as a transcriptional repressor either by simple competition with an activator for DNA binding known as passive repression or by recruitment of corepressors such as HDAC histone deacetylase to the promoter region and downregulate the target gene transcription. On Sp1, which can autoregulate its own promoter, KLF4 binds a sequence overlapping the Sp1 binding site and displaces Sp1 from its promoter element resulting in repression of the target gene [184]. In addition, KLF4 interacts with and recruits co-repressors HDAC1 and HDAC2 histone deacetylase to the CD11d promoter (leukocyte integrin gene) [185] and HDAC3 to the cyclin D1 promoter and represses their transcriptional activity [183]. KLF4 also represses Cytochrome p450 1A1 (CYP1A1) and cyclin gene such as cyclin D, Cyclin B1, and cyclin E [186]. These studies indicate that KLF4 can act as activator and repressor of transcription depending on promoter and cellular context of the target gene.

Expression of KLF4 is highly regulated and is stimulated upon various stimuli such as serum starvation, contact inhibition, DNA damage, sodium butyrate and oxidative stress [187]. KLF4 expression was induced in response to DNA damage and mediates G1/S phase cell cycle arrest in p53 dependent manner. KLF4 has been shown to physically interact with p53 and transactivates the cyclin-dependent kinase (cdk) inhibitor *p21WAF1/CIP1*, which binds to G1 cyclin-cdk complexes and inhibits DNA synthesis [188]. In addition, endogenous expression of KLF4 in cultured cells has been shown to inhibit DNA synthesis and cell proliferation [175]. These results indicate that KLF4 can act as a negative regulator of cell cycle and cell proliferation.

1.6.4.3. Expression and function of KLF4 in Smooth muscle cells

KLF4 is expressed at low levels in normal VSMC whereas its expression gets rapidly induced in rat carotid arteries following balloon angioplasty and in dedifferentiated vascular smooth muscle cells [139, 167, 189, 190]. KLF4 is a pleiotropic transcriptional regulator having both activating and repressing actions on gene expression [191]. KLF4 interacts with p65, the NF-κB subunit and transactivates inflammatory genes, but also competes with TGF-β effectors Smad3 for its coactivator p300 and inhibits TGF-β induced anti-inflammatory signaling. [192]. Likewise, KLF4 exhibits pleiotropic effects (anti proliferative and anti differentiation effects) on VSMCs [193]. KLF4 recruits HDAC2 and suppresses myocardin induced activation of CArG dependent SMC marker genes through induction of CArG hypoacetylation which causes decrease SRF binding to CArG elements and smooth muscle marker transcription [138, 167] (Fig. 1.5). In VSMCs, KLF4 participates in growth arrest, through induction of p53 expression, followed by upregulation of p21 $^{WAF/Cip1}$, a suppressor of cell cycle progression [194], suggesting that KLF4 has a pleiotropic effect on VSMC proliferation and differentiation.

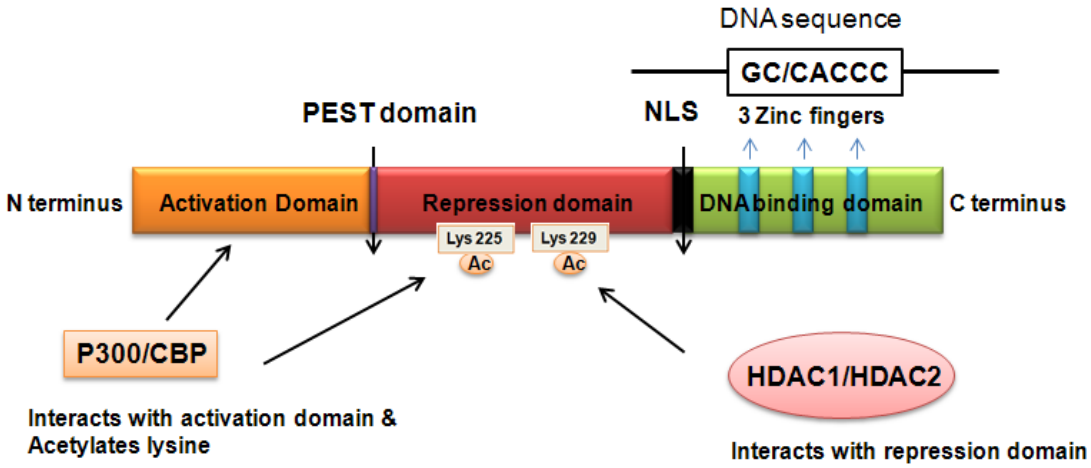


Figure 1- 4. Structure of KLF4 protein and its regulatory elements.

KLF4 has three domains, activation domain, repression domain and DNA binding domain. The coactivators like p300/CBP proteins and corepressors like HDAC1 and HDAC2 histone deacetylase interacts with the activation domain and repressor domains of KLF4 respectively. Acetylation of lysines at 225 and 229 position on repressor domain by coactivators' p300/CBP proteins is important for KLF4 mediated transactivation. The DNA binding domain has three Zinc fingers which bind to consensus (GC/CACCC)DNA sequence.NLS indicates nuclear localisation signal and PEST indicates (proline (P),glutamine (G),serine (S) and threonine (T)) sequence – signal peptide for ubiquitin-mediated proteasomal degradation.

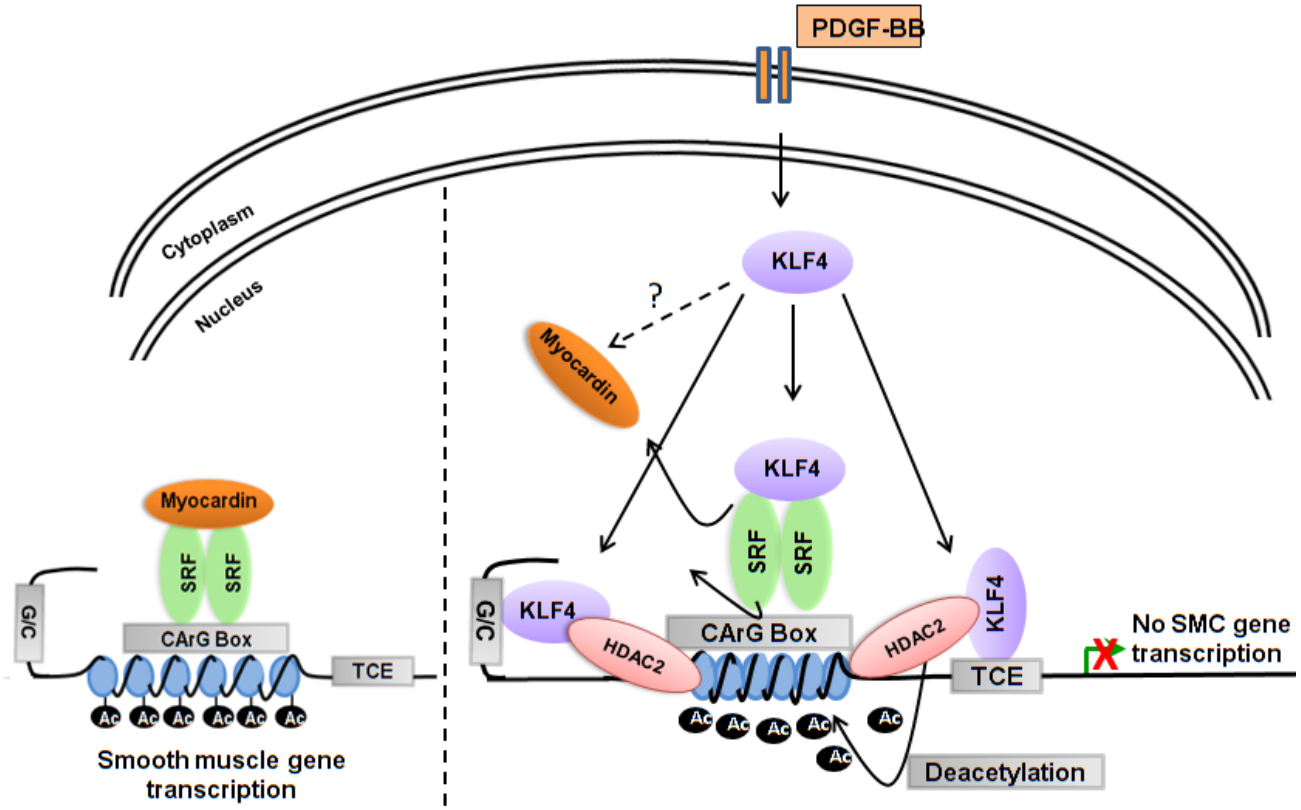


Figure 1- 5. Mechanism of KLF4 mediated transcriptional repression of smooth muscle genes.

A schematic model of transcriptional regulation of SMC genes is shown in the left panel. Transcriptional regulation of smooth muscle gene transcription is involves SRF and myocardin complex binding to CArG element within the promoter of smooth muscle genes. The right hand side of the figure explains the KLF4 mediated repression of smooth muscle gene transcription. PDGF-BB induces KLF4 expression during vascular injury. KLF4 binds to TCE and GC region within the smooth muscle promoters and recruits HDAC2 (histone acetylases), which induces histone hypoacetylation within the CArG element which displaces SRF/myocardin complex binding to CArG element. Thus KLF4 act as a negative regulator for smooth muscle gene transcription.

Of significance, the first seven base pairs (GAGTGGG) of TCE consensus sequence contains binding motif for KLF4 that is completely conserved in both rat and mouse SM22α and SM α actin promoters [139]. KLF4 was identified as a trans-acting factor that binds to TCE regions within SM22α and SM α actin promoters based on yeast one hybrid cloning analysis and electrophoretic mobility shift assays [139]. KLF4 binding to TCE regions negatively regulates TGF-β1-induced SM gene transcription whereas KLF5, another KLF family transcription factor, induces rather than represses TGF-β1 mediated SM promoter function [139]. Ectopic expression of KLF4 eliminates TGF-β1 induced SM22α and SM α actin promoter activity. However, TGF-β1 (2.5ng/ml) stimulation decreased the endogenous expression of KLF4 in cultured SMCs with no detectable KLF4 binding on the TCE region. [139]. These studies demonstrate that KLF4 act as a TCE-repressor for SMC differentiation, TGF-β1 downregulates KLF4 expression and transactivates SM gene expression.

KLF4 is regulated by multiple posttranslational modifications including phosphorylation [195], acetylation [183], sumoylation [196, 197] and ubiquitination [198]. KLF4 is degraded by ubiquitin-mediated proteolysis in response to TGF-β1 induced transcriptional activation [198]. TGF-β1 signaling pathway is also closely regulated by proteolysis including its own downstream modulators TGF-β receptors and Smads [199]. Several studies reported the role of E3 ligases including Smurfs [200], cdh1/Anaphase promoting complex (APC) [201] and Carboxyl terminus of hsp70-interacting protein**)** ligase, CHIP [202] in regulation of TGF-β1 signaling pathway. TGF-β1 (2ng/ml) rapidly downregulates the expression of KLF4 without altering its mRNA level in Mv1Lu (mink lung epithelial cells) cells. Furthermore, KLF4 has two conserved destruction boxes for cdh1/APC E3 ligases. Exogenous expression of cdh1 has shown to downregulate the expression of KLF4 protein and facilitates ubiquitination. Mutation analysis in these destruction boxes attenuates both cdh1 and TGF-β1 mediated KLF4 downregulation. These studies suggest that KLF4 is tagged by polyubiquitin catalysed by cdh1/APC E3 ligases and targeted for degradation in response to TGF-β1 induced transcriptional activation [198].

1.7. Roles for miRNA in SMC differentiation.

MicroRNA (miRNA) is short (~ 21-25bp) non coding RNA molecules that act as post transcriptional regulators in eukaryotes. The miRNAs bind to complementary sequences in the 3'UTR region on target mRNAs, results in degradation and mediates translation repression of target gene expression [203, 204]. The miRNAs are transcribed as longer hairpin loop primary transcripts and

processed by the RNase enzyme complex Drosha-DGCR-8 followed by dicer to yield mature single stranded miRNAs [205]. Recently, many studies have revealed key roles for miRNA in the control of cardiac [206] and smooth muscle development and remodelling upon vascular injury[207]. Among these, the miR-143 and miR-145 are highly specific to adult SMC lineage and involved in maintenance of contractile SMC phenotype [208]. The miR 143 /145 genes lie within 1.7 kb on mouse chromosome 18 and transcribed as a single bicistronic precursor with no homology between them, suggesting that they can act on different target sequences [209]. miRNA microarray hybridization assays in SMC enriched organs revealed that miR 143/145 gene cluster expression was confined to SMC during embryonic development. During postnatal development, the miR 143/145 gene cluster expression is downregulated in the heart but persists in vascular and visceral SMCs [209]. However, the expression of miR 143/145 gets downregulated in neointimal lesions following vascular injury as well as in atherosclerotic plaques. Cordes *et al*., [208] provide seminal evidence suggesting that miR-143/miR-145 control SMC fate and plasticity. They demonstrated that miR-145 promotes VSMC differentiation through increasing the expression of myocardin protein and miR-143 suppresses the expression of Elk-1, a key competitor for myocardin binding to SRF on SMC genes.

Furthermore, putative binding regions for miR-145 were identified in the 3' untranslated region (3'UTR) of myocardin and KLF4. Similarly, putative binding site for miR-143 was identified in 3'UTR of Elk-1. Luciferase based 3'UTR reporter assays showed that miR-145 enhances myocardin activity while miR-143 and miR-145 represses the expression of Elk-1 and KLF4, respectively which mediates the highly proliferative synthetic SMC phenotype. These results demonstrate that SMC phenotypic modulation was regulated by SMC enriched microRNAs, miR-143 and miR–145. [208].

The miR143/145 gene cluster has a conserved CArG element located between –3.2 to -4.2 Kb upstream of the gene and has been shown to be regulated by SRF and myocardin [209]. The miR 143/145 double knockout (dKO) mice were viable but displayed thinning of vessel wall with increased synthetic SMCs in the vessel wall. In addition, the dKO mice showed drastic reduction in blood pressure and reduced vascular tone compared with wild type mice, suggesting that miR 143/145 regulates the actin dynamics and are necessary for normal contractility of arteries *in vivo* [209]. Emerging evidence has reported that TGF-β1 activates miR 143/145 in human coronary smooth muscle cells (hCASMC) [210]. TGF-β1 induces

p38 MAPK and Smad pathways which then synergistically activates miR 143/145 expression in Smad, myocardin, SRF-CArG dependent manner. Of note, TGF-β1 induced myocardin and SRF expression gets downregulated by p38 MAPK inhibitor SB 203580 treatment, siRNA mediated knockdown of myocardin and SRF abrogated the TGF-β1 induced miR-143/145 expression. These results demonstrate a novel mechanism underlying TGF-β1 mediated SMC differentiation [210].

Recently, TGF-β family growth factors TGF-β1 and BMP4 were found to repress the expression of KLF4 through induction of miR-143/145 in VSMCs. Both TGF-β1 and BMP4 induce miR-143/145 expression but utilize distinct SRF cofactors; BMP4 requires MRTF-A while TGF-β1 requires myocardin for activation of CArG dependent miR-143/145 transcription and repression of KLF4 expression [211] (Fig.1.6). BMP4 stimulation mediates Rho signalling which enhances actin polymerisation and translocation of MRTF-A into nucleus where MRTF activates CArG-SRF dependent transcription of smooth muscle contractile proteins [116, 212]. In contrast, TGF-β1 activates myocardin/Smad proteins which synergistically upregulates smooth muscle genes. TGF-β1 facilitates Smad binding to TCE region on smooth muscle marker promoters and myocardin to CArG-SRF and activates transcription of SM markers [211, 213] (Fig.1.6). Various studies have identified other candidate miRNAs involved in SMC differentiation and proliferation. For instance, miR-26a [214], miR-1 [215] and miR-10a [216] were shown to be upregulated in SMC differentiation phenotype whereas miR-21 [217] and miR-221/222 [218, 219] were found to be upregulated in SMC synthetic phenotype.

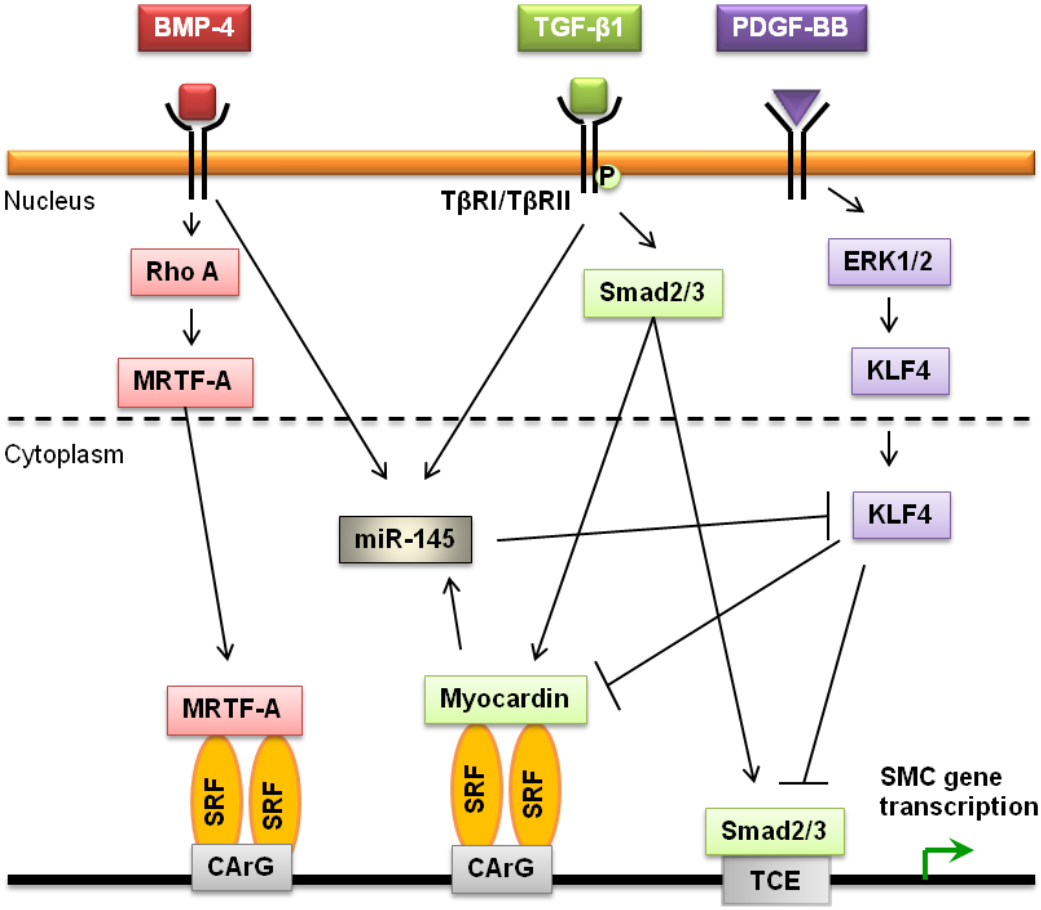


Figure 1-6. Signaling pathways involved in the regulation of KLF4 expression through induction of miR-145 expression in VSMCs.

BMP4 induce MRTF-A through RhoA signaling and TGF-β1 induces myocardin through Smad2/3 signaling.which enhances smooth muscle gene transcription in VSMCs. Both BMP4 and TGF-β1 signaling pathways induce miR-145 which downregulates KLF4 protein expression.

1.8. Cytokines

SMCs are not terminally differentiated but they retain remarkable plasticity undergoing reversible phenotypic changes in response to the local environment cues [75] which includes mechanical force [220], extra cellular components [221], reactive oxygen species [222], and cytokines like TGF β1 [223, 224], PDGF-BB and thrombin [225]. SMCs have diverse embryological origin which contribute to their heterogeneity in population [226].

SMC derived from neural crest and mesenchymal origin shows clear difference in serum independent growth properties in response to TGF-β1 treatment [227]. SMCs derived from neural crest were stimulated to proliferate following TGF-β1 treatment whereas this treatment inhibits mesenchymal derived SMC growth [227]. Similarly, PDGF-BB promotes SRF-dependent smooth muscle marker expression in proepicardial derived SMCs than those from other lineages [228], suggesting that the heterogeneity in SMC lineage is responsible for their lineage dependent variation in growth and differentiation properties upon growth modifiers.

1.8.1. TGF-β1

TGF-β1 belongs to the transforming growth factor beta superfamily of proteins including activins, inhibins, bone morphogenetic proteins which are involved in cellular proliferation, growth, differentiation and apoptosis [229]. TGF-β1 null embryos showed lethality around 10.5 day post coitus (dpc) with defects in yolk sac vasculature and endothelial development [229] indicates the critical role of TGF- β1 in promotion of differentiation during embryogenesis.

TGF-β1 has three isoforms, TGF-β1, TGF-β2 and TGF-β3 which are produced in dimeric latent forms and processed extracellularly to their active forms [230]. Endothelial cells, smooth muscle cells, macrophages and lymphocytes interact with TGF-β1 through the type I and type II transmembrane serine/threonine kinase receptors. TGF-β isoforms have high affinity for type II receptors. Binding of TGF-β on type II receptors, recruits the type I and forms complex, which results in phosphorylation and conformational change of type I receptor [230]. Further signal propagation to the nucleus occurs through the phosphorylation of Smads, TGF-β1 effectors by type I receptors. The Smads are classified into three types i) receptor regulated Smads (Smad1, Smad2, Smad3, Smad5 and Smad8), ii) Common Smad (Smad4) and iii) inhibitory Smads (Smad6 and Smad7) [231].

VSMC express many type I and type II receptors [232], whereas TGF-β1 signaling in VSMC is predominately mediated via Activin-like kinases 5 (ALK5)/TβRII

complex and subsequent signal propagation occurs through various signalling cascades including Smad 2/3, kinases such as p38 mitogen activated protein kinase (MAPK), p42/44, and Jun amino-terminal kinases (JNK) signalling cascades [233] [230].

TGF-β1 can act as a bimodal (inhibition and stimulation) regulator, depending on its concentration in SMC [234]**.** TGF-β1 in low concentration (1-2 fg/cell), stimulates cell proliferation whereas at higher concentrations, it inhibits cell proliferation in SMC [232]. Interaction between EC and SMC or EC and undifferentiated mesenchymal cells activates the latent TGF-β1, induces differentiation of mesenchymal cells to SMC/pericyte lineage and inhibits EC proliferation and migration [235], suggesting that effects of TGF-β1 vary depending on its concentration and cell type.

Mutation analysis of TGF-β1 revealed that nearly 50% of TGF-β1 homozygous and heterozygous mutant mouse embryos die *in utero* by E9.5 –E10.5 due to abnormalities in yolk sac with delayed vasculogenesis [229]. A similar embryonic lethality was exhibited in the TGF-β type II receptor null mutant mice [236]; suggesting that fetal death is due to lack of endothelial differentiation and capillary tube formation which occurred before the recruitment of VSMCs. There is emerging evidence to show that TGF-β1 can upregulate various smooth muscle differentiation markers in cultured SMCs [237, 238]. In addition, TGF-β1 has been shown to induce smooth muscle differentiation in neural crest cells as well as in neural cell derived Monc-1 cells [239]. Furthermore, co-culture studies of mouse embryonic multipotent 10T1/2 cells with endothelial cells have shown to induce SMC differentiation in 10T1/2 cells [240].

Sinha *et al* [241] demonstrate that mouse ESC-EB expresses SMC specific genes including SM α actin, SM22α, SMMHC, myocardin and smoothelin. However, adenoviral mediated attenuation of TGF-β type II receptor expression (which is specific to endogenous TGF-β1 expression) completely eliminates SM marker expression in embryonic stem cell (ESC)-derived embryoid bodies (EBs). Additional studies with siRNA repression of Smad2 and Smad3 have shown that the expression of SM α actin, an early SM marker is dependent on both Smad2 and Smad3 whereas the expression of SMMHC, late SM markers is dependent only on Smad3. These studies provide a direct evidence for TGF-β1 signaling through Smad2 and Smad3 in smooth muscle differentiation from embryonic stem cells but

also provide evidence for differential TGF-β-Smad signalling involved in expression of early vs. late smooth muscle genes [241].

1.8.2. PDGF-BB

Platelet derived growth factor (PDGF) family consists of three isoforms made from pairwise combination of PDGF A and PDGF-B chains named as PDGF-AA, PDGF-BB and PDGF-AB. Among the isoforms, PDGF–BB is a powerful chemoattractant and mitogen followed by PDGF AB and PDGF-AA. The PDGF receptor binds to similar ligands, which is made of dimers of α and β subtypes of the PDGF tyrosine kinase receptor. The α receptor binds both the A and B chains of PDGF whereas β receptor selectively binds only B chain of PDGF ligand. In the vascular wall, the main function of PDGF is the paracrine recruitment of mural precursor cells to the vessel wall and stimulation of endothelial cells proliferation by autocrine process [235].

PDGF-BB represses the expression of SM α actin and SMMHC and with prolonged treatment induces the phenotypic modulation in SMCs but has no effect on β-actin content [242]. However, withdrawal of PDGF-BB reversed the PDGF-BB induced repression, suggesting PDGF-BB acts as negative regulator for smooth muscle specific marker differentiation [243]. In contrast, SMC from proepicardial origin [244] or mononuclear cells [245] isolated from blood plated on collagen with PDGF-BB treatment favours the SMC differentiation; indicating that depending on SMC lineage PDGF-BB can act as positive or negative regulatory agent on control of SMC differentiation.

1.9. SMC differentiation from stem/progenitor cell sources

The variation in VSMC population and involvement of progenitor cells in vascular diseases necessitates improved understanding of the signaling pathways that induce differentiation of these vascular progenitor cells towards SMC specific lineage [224, 240, 246]. To date, several progenitor cells were capable of SMC differentiation *in vitro* cellular models have been identified from various sources including 1) embryonic stem cell and embryoid bodies 2) neural crest cells 3) proepicardial cells 4) mesoderm derived cells [247] and 5) smooth muscle stem cells and 6) smooth muscle progenitor cells based on the source of vascular progenitor cells (Table 1-1).

1.9.1. Embryonic stem cell (ESC) and embryoid bodies

ESC based models include p19 and A404 cell types. P19, an embryonal carcinoma cell line was derived from mouse teratoma formed by implanting 7.5 day embryos into testis [248]. P19 cells were induced to differentiate into fibroblast like cells expressing SM α actin on treatment with retinoic acid (RA–10^6 mol/L) for 48 hours. The differentiation of p19 cells into SMC showed less than 5% efficiency and these cells do not express other SM markers, highlighting the requirement of additional techniques to improve SMC differentiation. Use of the multipotent A404, clonal cell line derived from p19 increased SMC differentiation efficiency, up to 90% on treatment with retinoic acid (RA 1μM/L) and expressed SM markers such as SM α actin, calponin, SM1 isoform of SMMHC, the late differentiation marker and myocardin, suggesting that the A404 cell line model can be used to study the early differentiation of SMC development [246]. Furthermore, differentiated A404 cells showed higher levels of histone H4 acetylation within 5' flanking regions of SM α actin and 5' flanking and first intronic region of SMMHC promoters, whereas skeletal muscle promoters showed no acetylation in their promoter regions [246]. In addition, SMMHC promoter showed methylation of H3 histones specific to its 5' flanking CArG element and both H3 and H4 histone modification in its translational start site, suggesting that chromatin modification plays a major role in selective induction of CArG dependent SMC specific marker genes during development and differentiation [246].

Human ESC cultured in a monolayer with 10 mM all trans retinoic acid demonstrated SMC differentiation at an efficiency greater than 93% for SM α actin and SMMHC positive cells [249]. Several studies have demonstrated that stem cell surface markers can be used to isolate SMC progenitor cell subpopulations from ESC and enrich the SMC differentiation from embryonic stem cells [250]. Murine embryonic stem cell derived Sca-1^+ progenitor cells were induced to differentiate into SMC mediated by PDGF receptors and collagen IV α1, β1 and αV integrins [251] whereas vascular endothelial growth factor (VEGF) treatment promoted endothelial differentiation [252]. This model provided early evidence for the requirement of extracellular matrix collagen IV in early stage of smooth muscle differentiation. Embryonic stem cells grown on collagen IV (5μg/ml) promote early stage SMC differentiation as compared to collagen type I and fibronectin [251]. Moreover, collagen IV synthesis was detected by western blot in collagen I and fibronectin treated groups and also autocrine secretion of collagen IV in some cultured conditions has shown to enhance the spontaneous differentiation of embryonic stem

cells [251]. Embryonic stem cell derived Sca-1^+ cell culture on collagen IV with PDGF BB (10ng/ml) was reported to induce SMC differentiation in 55-66% of cells and long term selection in the presence of PDGF-BB and collagen IV increased the SMC differentiation to 95 % for SM markers such as SM α actin, calponin and SMMHC [251]. These results suggest that collagen IV plays a major role in SMC differentiation from embryonic stem cells [251].

Yamashita *et al*., [253] found that ESC derived Flk (Fetal Liver Kinase)-1^+ (Flk1 –receptor for VEGF) cells can differentiate into endothelial and mural cells. Flk-1^+ cells were obtained by culturing ESC on collagen IV coated dishes with 10% FCS for four days. Flk1^+ cells treated with VEGF 165 (50ng/ml) promotes PECAM *(*Platelet endothelial cell adhesion molecule) 1^+ sheets of endothelial cells whereas PDGF-BB (10ng/ml) has been shown to induce smooth muscle cells with expression of SM α actin, calponin, SMMHC and SM22α. Intracardiac injection of LacZ expressing Flk1^+ cells into stage16-17 chick embryo showed the Lac Z signal along the vessels in head, yolk sac, heart and intersomitic region, and immunostaining of cross sections showed colocalisation of PECAM1^+/nLacZ and SMA^+/nLacZ, suggesting that Flk-1^+ cells can differentiate into endothelial and mural cells *in vivo* and plays a role in to vascular development [253].

Embryoid body (EB) cultures are three dimensional aggregates of stem cells grown in suspension *in vitro* which can form all three germ layers, recapitulate early embryonic development [254] and can be used to study *in vitro* SMC development [255]. Retinoic acid (all-trans-Retinoic acid- ATRA-10^{-8} mol/L) and dibutyryl –cAMP ($0.5x10^{-3}$ mol/L) treatment of EBs obtained from mESCs induced 60% of spontaneously contracting SM-like cells with expression of SM α actin and SMMHC. In addition, these cells have been shown to respond to angiotensin II and exhibit expression of ion channels similar to VSMCs [255]. These results suggest that EBs may be a suitable model to study in vitro SMC differentiation. A major limitation of this model is the lack of selective techniques for isolating the differentiated SM like cells from the EB for further investigation [241, 247, 255]. Additional enrichment strategies were developed to eliminate the disadvantages of the EBs model using a stably expressing puromycin resistance gene driven by SMC specific promoters to enhance the enrichment of SMC from ESC-EBs [256]. These differentiated SMCs expressed SMC specific markers, SM α actin and SMMHC and showed agonist induced calcium transients whereas subcutaneous injection of these differentiated SMCs has been shown to develop teratoma within 3 weeks of administration [256]. Increasing the puromycin selection time (prior to injection for 3

days) eliminates the teratoma formation but fails to develop mature blood vessels *in vivo* [256]. Furthermore, enrichment for stem cell surface marker CD 34 has shown high efficiency for SMC differentiation [257]. Ferreira *et al*., [257] reported that hESC can be differentiated towards SMC and endothelial cells. The endothelial/hematopoietic marker, CD 34 was used to isolate the vascular progenitor cells from EBs. Treatment of $CD34^+$ cells with VEGF -165 (50ng/ml) and PDGF (50ng/ml) induces endothelial like (EL) and smooth muscle like (SML) differentiation respectively. The smooth muscle like cells express SM markers including SM α actin, calponin, SMMHC and SM22α and also showed contraction in response to carbachol. Subcutaneous administration of EL and SML cells separately or in 3:1 ratio results in development of vasculature in nude mice. These studies provide evidence that the EB model can recapitulate early embryonic development and can be used to study the SMC development, but presence of different cell types and low permeability for soluble factors to contact the inner cells makes it a less efficient model to study SMC differentiation mechanism [256, 258].

1.9.2. Neural crest cell model

Pluripotent neural crest cells can differentiate into neurons, chondrocytes, glia, melanocytes and smooth muscle cells whereas the immortalized neural crest cell line Monc-1, has shown efficient differentiation to smooth muscle cells [224]. Vascular smooth muscle cells in the cardiac outflow tract arise from a neural crest origin and *in vivo* differentiation of neural crest cells to VSMC is mediated by TGF-β1 signaling [259]. Freshly isolated rat neural crest stem cells differentiate into SMC with expression of SM markers(SM α actin, calponin and SMMHC) in the presence of TGF-β1 (20 pM) and subsequent activation of the calcineurin signalling pathway [259]. The $p75^+$ murine neural crest cell line was immortalised by retroviral transfection of *v-myc* gene in their primary culture and further clonal selection generated multipotent mouse neural crest cell line, Monc-1 cell lines [260]. The Monc-1 cells differentiate to smooth muscle cells in the presence of TGF-β1 (5ng/ml) after three days in culture [239]. The differentiated SMC showed spindle shaped morphology with expression of SM markers such as SM α actin, SM22α, calponin, SM γ actin, smoothelin and SMMHC and contracts in response to a muscarinic agonist carbachol. In addition, TGF-β1 downregulates E-cadherin gene expression and withdrawal of TGF-β1 in culture media did not result in loss of VSMC markers expression indicating that Monc-1 cell lines may be used to study SMC development [239]. In spite of the fact that Monc-1 cell line can yield a stable SMC

phenotype on TGF-β1 treatment, the requirement of a complicated media for maintenance of its undifferentiated state [261] is a major disadvantage of this model. Likewise, JoMa1 is an immortalised neural crest stem cell line obtained from transgenic mouse having conditional tamoxifen inducible expression of c-myc oncogene. Removal of tamoxifen and TGF-β1 (1ng/ml) for six days has reported to induce SMC differentiation with SM α actin, SM γ actin and calponin which suggest that JoMa1 line can be induced to differentiate towards SMC by TGF-β1 treatment and an attractive model to study SMC development whereas this model has limitations similar to Monc-1 neural crest stem cell line in maintaining its undifferentiated state [262].

1.9.3. Proepicardial cells

Landerholm *et al*., [244] reported the requirement of functional serum response factor for differentiation of proepicardial cells from Hamburger-Hamilton stage 17 quail embryos to coronary smooth muscle cells (CoSMC). The proepicardial cells attain the epithelial to mesenchymal transition with calponin, SM α actin and SM γ actin expression within three days and by sixteen days; they express complete smooth muscle morphology with upregulated smooth muscle markers including SMMHC, SM α actin, calponin and SM γ actin. The expression of a dominant negative SRF construct blocks the expression of smooth muscle positive cells whereas it has no effect on epithelial to mesenchymal transition; suggesting that functional SRF is necessary for the differentiation of proepicardial cells to CoSMC [244].

1.9.4. Mesoderm derived model

The mesoderm model includes 10T1/2 cell line derived from mouse C3H embryo cells. The mouse C3H embryo cell line was established from 14-17 days old whole mouse embryos. A C3H mouse embryo cell line which exhibited high degree of post confluent inhibition of cell division was designated as C3H/10T1/2 otherwise called as 10T1/2 cell line [263]. Multipotent 10T1/2 cells can be differentiated towards adipocyte, osteoblast and myoblast cell lineages whereas in co-culture with endothelial cells, they differentiate towards smooth muscle lineage, suggesting that endothelial cells can induce the differentiation of undifferentiated mesenchymal cells towards SMC like cells during vasculogenesis and angiogenesis *in vivo* [240]. In addition, proliferating endothelial cells release various diffusible growth factors such as PDGF and fibroblast growth factors (FGF) in their local environment, which act as chemoattractants for SMC during vessel formation [264, 265]. Both the EC and

10T1/2 cells produce latent TGF β1 in their culture conditions. The co-culture environment may activate TGFβ1 which directs the differentiation of 10T1/2 cells towards SMC lineage [240]. Of interest, multipotent 10T1/2 cells when treated with TGF-β1 (1ng/ml) express SM α actin, calponin, SMMHC and SM22α [240]. In contrast, myocardin, a transcriptional coactivator specific to SMC and a marker to distinguish differentiated SMC from activated fibroblast [226] was not expressed in TGF-β1 treated 10T1/2 cells [94], suggesting that 10T1/2 cells cannot be used as a definitive model to study SMC differentiation.

Aortic endothelial cells can be induced to SM like cells on treatment with TGF-β1 (1ng/ml) for 10 days [266]. The differentiated SM like cells comprise a heterogeneous population of SMC cells such as contractile and synthetic SMCs indicating that during atherogenesis TGF-β1 released at the injury site can induce endothelial cells to differentiate into SMC. This model can be used to study the pathological events contributing to atherogenesis [266].

In summary, the heterogeneity of VSMCs is due to their diverse developmental origin, which contributed for establishment of different *in vitro* cellular models to investigate their role in vascular development and diseases. These models suggest that SMC development is regulated by different growth factors depending on their source of origin. Hence, the selection of model and growth factors for differentiation is dependent on the development origin of SMCs.

Table 1-1. Sources of stem cells having a differentiation potential towards SMC

S.No.	Cell model	Cell type	Cytokine/ Growth factor or Extracellular matrix	Result
1	ESC and embryoid bodies	P19	RA–10^{6} mol/L	Induce 5% SMC differentiation [246].
2		A404	RA 1μM/L	Induce Up to 90% SMC differentiation with SM α actin, calponin, SMMHC marker expression [246].
3		Human ESC	10 mM all trans retinoic acid	Induce >93% SMC differentiation with SM α actin and SMMHC expression [249].
4		Murine ESC derived Sca-1^{+} progenitor cells	PDGF BB (10ng/ml) with collagen IV (5μg/ml)	Induce 55-66% SMC differentiation [251].
5		hESC derived $CD34^{+}$	PDGF (50ng/ml)	Induce SMC like cells expressing SM α actin, calponin, SMMHC and SM22α [257].
6		Embryoid body (EB) cultures	All-trans-Retinoic acid-(ATRA)-10^{-8} mol/L	Induce 60% of spontaneously contracting SM-like cells [255].
	Neural crest cell model			
7		Rat neural crest stem cells	TGF-β1 (20 pM)	Differentiate into SMC with expression of SM markers [259].
8		Monc-1 cells	TGF-β1 (5ng/ml)	Induce spindle shaped SMC like cells expressing SM markers [239].
9		JoMa1 cells	TGF-β1 (1ng/ml)	Induce SMC differentiation expressing SM α actin, SM γ actin and calponin [262].

10	Proepicardial cells			Differentiate into coronary smooth muscle cells (CoSMC) in culture [244].
11	Mesoderm derived model	EC and 10T1/2 cells coculture		Induce differentiation of 10T1/2 cells towards SMC lineage [240].
12		10T1/2 cells	TGF-β1 (1ng/ml)	Induce SMC like cells [240].
13	Smooth muscle stem cells	Bone marrow derived stem cells in circulation		Participate in lesion development [276, 279, 282].
14		Bone Marrow derived Mesenchymal Stem Cells (BMMSC)	Laminin coated plate	Differentiate into SMC with expression of SM α actin and calponin [285].
15		BMMSC	TGF-β1 or in combination with ascorbic acid	Induce SMC differentiation with expression of SM markers like SM α actin, calponin and SM22α [287].
16	Adventitial progenitor cells	Sca-1 $^{+}$ cells from adventitia	PDGF-BB (10ng/ml)	Differentiate into SMC [250].

1.10. Smooth muscle stem cells

VSMC are heterogeneous and retain remarkable plasticity in culture [272]. Part of SMC variation can be explained by their diverse embryological origin [247]. For example, the neural crest cells derived VSMCs are located in pharyngeal arch arteries including aortic arch, left and right carotid arteries, ascending aorta [273, 274] whereas the descending aorta VSMC originate from somites [275, 276]. The proepicardial-derived VSMCs are present in coronary vessels and the base of the aorta [273, 277] and pulmonary trunk VSMCs are derived from secondary heart field [278, 279].

In response to vascular injury, SMCs attain a synthetic state with a high rate of proliferation, migration and production of extracellular components, and contribute to the development of various vascular diseases [105]. Initially it was considered that the intimal SMCs within atherosclerotic lesions migrate from medial SMC (local origin) to intima during vascular injury [280]. However, recent studies, suggests that vascular progenitor cells were present within blood circulation [268, 281, 282], bone marrow [267] and from adventitial fibroblast [283], and contribute to postnatal neovascularisation [284]. The smooth muscle progenitor cells have the ability to differentiate into SMCs when cultured in the proper conditions. Therefore, the knowledge of a signalling mechanism controlling the SMC differentiation and origin of SMC during neointimal formation is important in understanding the vascular injury induced SMC accumulation and subsequent arterial remodelling.

1.10.1. Bone marrow derived stem cells

Bone marrow contains two distinct stem cell types, hematopoietic (which produce blood) and mesenchymal stem cell (which produce stromal and bone cells). Apart from blood cells, hematopoietic stem cells (HSC) have a potential to differentiate into many cell types including hepatocytes, epithelial cells and cardiomyocytes [272]. Several line of evidences confirmed the presence of bone marrow derived SMC progenitor cells in lesion development and tissue repair process [267-269]. Sata *et al*., [268] reported evidence of vascular smooth muscle regeneration from bone marrow derived cells using the experimental models including atherosclerosis, post angioplasty and graft vasculopathy. These models showed that whole bone marrow labelled cells contribute to ~25-50% of the SM α actin positive smooth muscle like cells within the neointimal and medial layers in injured artery. Studies using aortic transplants into β-galactosidase transgenic recipients showed stable integration of LacZ positive marrow cells expressing SM α

actin within the medial layers of the vessel, suggesting that smooth muscle like cells can originate from circulating bone marrow derived stem cells [267]**.**

In a chimeric mouse model, bone marrow derived from a male donor was transplanted into irradiated female mice, 56% of neointimal cells were shown to react with Y1 chromosome probe suggesting a recipient origin [269]. The circulating bone marrow cells were recruited only after severe vessel damage and resembled fetal/immature vascular smooth muscle cells, suggesting that severity of injury plays a role in recruitment of bone marrow cells [269]. Participation of circulating bone marrow in vascular repair and lesion development was also reported in non-irradiated parabiotic mouse model. When wild type mice were conjoined subcutaneously with green fluorescent protein (GFP) transgenic mice, ~15% of neointimal and ~32% of medial cells were positive for GFP and showed SM α actin expression. The contribution of marrow cells in human coronary atherosclerosis was demonstrated using sex mismatched marrow transplantation. The coronary atherosclerotic plaques from gender mismatched bone marrow transplanted patients showed smooth muscle cells from donor origin [282]. In an effort to determine which cell type in heterogeneous bone marrow contributes to vascular remodelling, highly enriched HSC (stem cell antigen-1 ($Sca\text{-}1^{+)}$, $c\text{-}kit^{+}$, Lin^{-}) population was isolated from bone marrow of Lac Z mice and transplanted into wild type. Upon wire injury of the femoral artery these cells showed neointimal hyperplasia to the same extent as whole bone marrow [285]. The neointimal and medial region contains many LacZ positive cells (~33%) and expresses SM α actin. The LacZ positive cells also contribute to re endothelialization which suggest that bone marrow enrichment for $Sca\text{-}1^{+}$, $c\text{-}kit^{+}$, Lin^{-} fraction homing the injured vessel have a tendency to differentiate into either SMC or EC which may contribute to vascular remodelling [285]**.** Taken together, these studies suggest the role of circulating bone marrow derived stem cells contributing to neointimal formation and lesion development after vascular injury.

1.10.2. Bone Marrow derived Mesenchymal Stem Cells (BMMSC)

Mesenchymal stem cells (MSCs) are multipotent stromal cells that have the ability to differentiate into osteoblasts, chondrocytes and adipocytes [286]. BMMSC cultured on laminin coated plates for seven days induced SMC differentiation and expressed SMC specific proteins such as SM α actin and calponin. Furthermore implanted BMMSC on laminin sheets in *in vivo* for two weeks showed an increased expression of SM α actin, calponin and SM2 isoform of SMMHC [270]. Several studies showed that the ERK/MAPK pathway is

antimyogenic in SMC and involved in activation of growth related genes mediated by phosphorylation of Elk-1 gene [287]. Inhibition of MEK/ERK signaling by the MEK inhibitor PD98059 induced myocardin, the transcriptional coactivator and SM markers like SM α actin, SMMHC and caldesmon in differentiated BMMSCs. However, MEK inhibitor induced differentiation can be reversed by PDGF-BB treatment which indicates that PDGF-BB is a strong activator of MEK/ERK signalling pathway and its inhibitory effect on SMC is mediated by activation of MEK/ERK signalling pathway [287]. BMMSC cultured in the presence of TGF-β1 or in combination with ascorbic acid induced SMC differentiation with expression of SM markers like SM α actin, calponin and SM22α [271].

1.10.3. Adventitial progenitor cells

Several studies have found evidence for the possibility of non bone marrow derived progenitor cells. It has been reported that myofibroblasts migrate from the adventitia and participate in the neointimal formation in response to vascular injury [288]**.** Hu, *et al.,* [250] reported a source of smooth muscle progenitor cells in ApoE deficient mice that contributes to atherosclerotic lesion development. They have identified Sca-1 $^{+}$ (21%), c-kit $^{+}$ (9%), CD34^{+} (15%) and Flk-1 $^{+}$ (4%) cells in the adventitia of aortic roots. The Sca-1 $^{+}$ cells were differentiated into SMC *in vitro* in the presence of PDGF-BB (10ng/ml). Moreover, the Sca-1^{+} expressing LacZ gene transplanted into adventitia of Apo E deficient mice showed an accumulation of β-gal positive cells in the neointima and enhanced the development of atherosclerotic lesion [250].

Other than bone marrow, circulating blood and vessel adventitia, there are other sources of progenitor cells in the body including skeletal muscle [289] and heart [290]**.**

1.10.4. Risk associated with stem cell based therapy

Stem cells have an ability to replicate indefinitely and remain undifferentiated in culture. One of the hall marks of stem cells is the formation of non cancerous tumor named as teratoma *in vivo* [291]*.* Stem cells are widely used to study the normal molecular mechanism associated with disease development as well as drug discovery for regenerative medicine. However, *s*tudies with the introduction of stem cell into animal models fail due to teratoma formation [292]. A recent study reported the malignant tumor-inducing capacity of hESCs including H1 and Human embryonic stem cells (HSF6) [293]. These results emphasized a need for

development of improved techniques to enhance the safety of stem cells in regenerative medicine.

The main approach to enhance the safety of stem cell for regenerative medicine is to differentiate stem cell into progenitor cells, a level below in the hierarchy. Furthermore, these differentiated cells are subjected to sorting either for the progenitor specific markers or for the stem cell markers to eliminate the undifferentiated stem cells prior to implantation into animal [291]. These progenitor cells were reported to exhibit low immunogenicity in their undifferentiated state, which can be implanted without much immunogenic alterations as compared to ESC and multipotent stem cells [294].

1.11. Smooth muscle progenitor cells

A number of fate mapping studies have identified several origins for smooth muscle progenitor cells [295, 296]. Although these progenitor cells have a different origin they transcribe a common set of markers such as SM α actin, calponin, SMMHC and SM22 α on differentiation [297]. Several findings suggest that a smooth muscle progenitor cell resides within the adventitial and the medial layer [297].

The progenitor phenotype in these cells are maintained through transcriptional silencing of SRF dependent smooth muscle markers either by chromatin modification of CArG elements [298-300] or inhibition of SRF binding to CArG elements [127, 164, 167, 297, 301]. Furthermore, the silencing of SM markers in smooth muscle progenitor cells is enhanced through interaction of SRF-CArG elements with potent transcriptional repressors [297]. Passman and coworkers [302] have identified coexpression of repressors such as Msx1, KLF4 and Forkhead transcription factor 4 (Foxo 4)with SRF and myocardin in $Sca1^{+}$ smooth muscle progenitor cells isolated from aortic adventitia. In addition PRISM (PR domain in smooth muscle) (*prdm6*) protein is reported as a transcriptional repressor in smooth muscle progenitor cells. PRDM 6 recruits histone methyl transferases and class I HDAC to myocardin and GATA 6 and further downregulates the SMC gene transcription in smooth muscle progenitor cells [297, 303].

1.11.1. Circulating smooth muscle progenitors cells

Several studies suggest that bone marrow derived lineage committed cells can differentiate into SMCs. Simper *et al*., [245] provide early evidence for the existence of circulating smooth muscle progenitor cells within human blood. They showed that progenitor cells within the mononuclear cell pool isolated from

peripheral buffy coat from human blood when grown on collagen type I matrix for three weeks in the presence of PDGF-BB (50ng/ml) can differentiate into smooth muscle outgrowth cells (SOCs). These SOCs expresses smooth muscle markers (SM α actin, Calponin and MHC) and CD34, Flk-1 and fms-related tyrosine kinase 1 (Flt-1), but not Tie-2, suggesting that SOCs might have originated from bone marrow source distinct from Tie-2 receptor positive EOCs. These outgrowth cells are highly proliferative (>40 population doublings) and have markedly increased α5 and β1 integrin subunit protein expression which is a characteristic feature of the proliferative SMC phenotype [245]. These results suggest that smooth muscle progenitor cells reside within circulating mononuclear fraction differentiate into SOCs and have a potential for proliferation at sites enriched in extracellular matrix deposition. Furthermore, the clonally expanded high proliferation potential SMC outgrowth cells (HPP-SOCs) are positive for the myeloid marker CD 14 and retained the CX3CR1 chemokine receptor. The CX3CR1 subpopulation of bone marrow cells isolated from CX3CR1 recombinant $^{gfp/+}$ transgenic mice differentiates into smooth muscle cells in the presence of smooth muscle differentiation media with PDGF-BB (100ng/ml) [304]. In addition, bone marrow derived $CX3CR1^{+}$ progenitor cells have shown to differentiate into SMC mediated through fractalkine interaction and reported to contribute 5-10% of neointimal SMC following mild wire injury [304]. These studies suggest that fractalkine-CX3CR1 chemokine receptor is critical for the SMC recruitment and neointimal formation followed by vascular injury.

Aortic and cardiac allograft implanted in rats results in the development of transplant arteriosclerosis characterised by perivascular inflammation and neointimal thickening, composed of SM α actin positive vascular smooth muscle cells. Using a MHC class I haplotype specific immunohistochemical technique and PCR analysis showed that neointimal SM α actin positive cells were from recipient origin and not of donor origin [305]**.** The CD14/CD105 double positive peripheral blood mononuclear cells (PBMNC) subpopulation but not the CD14/CD105 negative isolated from peripheral blood differentiated into smooth muscle like cells (SMLC) when cultured for long term in the presence of growth factors (PDGF-BB 5ng/ml, b-FGF – 5-10 ng/ml and TGF-β1 2ng/ml). Peripheral blood-derived SMLCs were positive for smooth muscle markers including SM α actin, calponin and SMMHC [306]. Furthermore, the expression of CD14/CD105 double positive cells was found to be elevated in atherosclerotic patients. These results suggest that CD14/CD105 double positive PBMNCs acts as a source for human smooth muscle precursor cells in circulation and participate in the pathogenesis of vascular diseases [306]**.**

However, controversy exists relating to the origin of smooth muscle progenitor cells recruitment in neointimal formation and vascular lesion development.

1.12. Isl1^{+} (LIM-homeobox transcription factor positive) progenitor cells

Numerous studies identified a rare subset of the LIM-homeobox transcription factor positive (*Isl*1^{+}) progenitor cells in the atria, outflow tract and right ventricle of mammalian heart after birth [307]. A multipotent *Isl*1+ cardiovascular progenitor cells (MCIPs) develops various cardiovascular cell types *in vivo* during embryonic development. The triple positive (*Isl*1^{+}, *Nkx2.5*$^{+}$, and *Flk*1^{+}) embryonic derived cardiovascular multipotent precursors differentiated into cardiac muscle, smooth muscle and endothelial lineages *in vitro.* The *Isl*1^{+} cells expressing LacZ genes differentiated into smooth muscle cells either alone or co-cultured with human coronary smooth muscle cells [308]. In support of this concept, multipotent *Flk*1^{+} and *bry*$^{+}$/*Isl*1^{+} isolated from embryonic stem cells differentiate into cardiomyocytes, endothelial and vascular smooth cells [309], Multipotent *Isl1*$^{+}$ cardiovascular progenitor cells created from mouse and human iPS cells when injected directly into the left ventricular wall of nude mice they successfully engraft into host tissue without teratoma formation [310]. Furthermore, the engrafted cells differentiated into all three cardiac (cardiac muscle, smooth muscle and endothelial) lineages at the site of injection [310].

*Isl*1 null mice exhibited growth retardation and die between E 10.5 –E 11 with severe defects in cardiac phenotype. *Isl*1 knockout hearts fail to undergo looping morphogenesis and have a single atrial and ventricular chamber [311]. Moreover, both right ventricle and outflow tract are absent in *Isl*1 homozygous mice. These results suggest that *Isl*1 is expressed in second heart field and contribute to venous and arterial formation in the heart [311]. In addition, lineage tracking of *Isl*1 positive cells revealed the homing of these cells in the outflow tract, the right ventricle and in part of atria and left ventricle region which indicates that *Isl*1 transcription factor marks cardiac progenitor cells of second heart field [312].

The colonisation of *Isl1* progenitor cells in the embryonic structures such as outflow tract and inter-atrial septum suggest that these cells might participate in vascular remodelling in response to vascular injury. Thus, *Isl1*$^{+}$ cardiovascular progenitor cell model is a powerful tool for studying signalling pathways responsible for cardiovascular disease development as well as for designing a cell based assay to identify new therapeutic targets and pathways for treatment of cardiovascular diseases.

1.13. Human smooth muscle stem progenitor cells

Our group has recently isolated novel *Isl*1^{+} stem progenitor cells from leukocyte filters of atherosclerotic patients undergoing coronary artery bypass grafting (CABG) surgery and from the peripheral blood of patients with pulmonary hypertension. These cells express stem cell markers such as Octamer-binding transcription factor 4 (Oct 4), sex determining region Y)-box 2 (Sox-2), KLF4, stage-specific embryonic antigen 1 (SSEA-1) and c-kit. Human smooth muscle stem progenitor cells (hSMSPCs) are clonogenic (Fig.1.7 A), with high self renewal potential and exhibit high level of telomerase activity (Fig. 1.7 B) and high population doubling more than 140 population doublings (Fig. 1. 7 C). However, hSMSPC cells express smooth muscle markers SM α actin (Caplice *et al*., unpublished data) at a modest level as measured by FACS analysis. Since these cells were positive for stem cell and smooth muscle markers, these cells were named as human smooth muscle stem progenitor cells (hSMSPC).

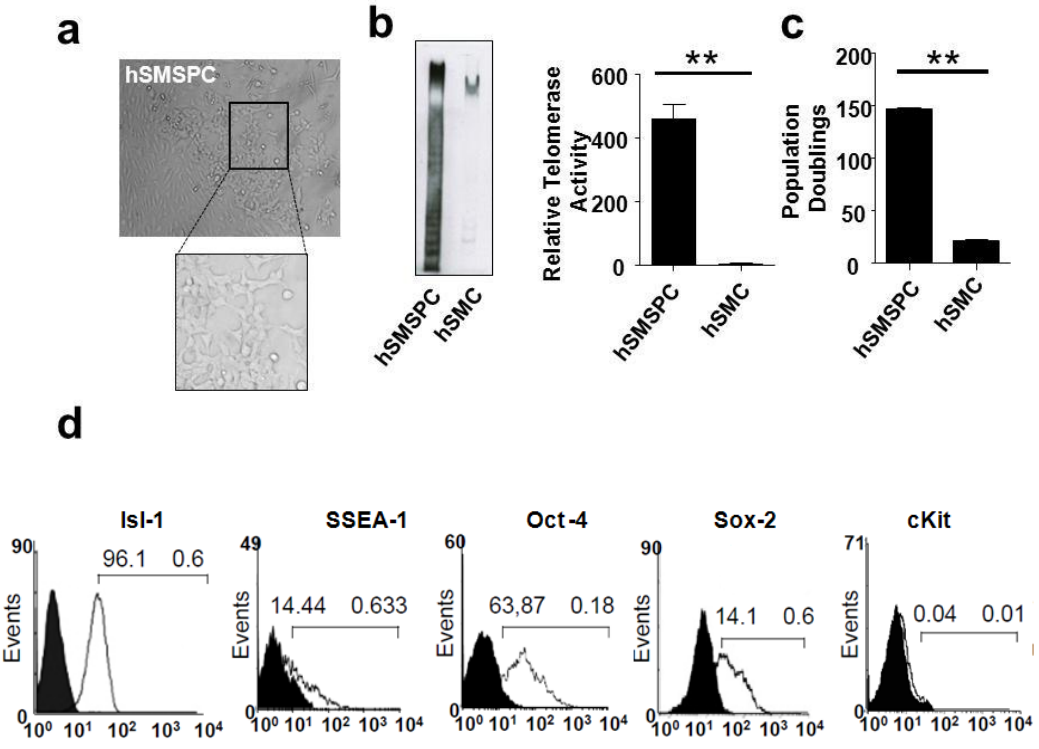


Figure 1-7. Phenotypic characterisation of hSMSPC cells.

A) To prove that hSMSPC cells were clonogenic, hSMSPC cells were transduced with lentiviral vector driving eGFP expression (HR-CSGW). hSMSPC cells were capable to form a colony from single cell. B) hSMSPC cells exhibit high level of telomerase activity and high population doublings (C) as compared to SMCs. D) hSMSPC cells express Isl1, stem cell markers SSEA-1, Oct 4, SOX-2 and ckit as measured by FACS analysis (Caplice et al., unpublished data).Data are representative of three independent experiments. **P<0.01.

1.14. Clinical significance of adult stem cells

Atherosclerosis is a leading cause for coronary artery disease caused by narrowing of artery due to lipid accumulation [313]. Artery from patients (elsewhere) is grafted to the coronary artery to bypass atherosclerotic occlusions (autografts). The main challenge facing the treatment of disease artery is the limited availability of autografts from patients [314]. This led to the investigation of cell based therapeutic approaches to regenerate the vascular grafts. In an attempt to replace the injured vessel wall, cellular based therapy has been extensive investigated in the past decade to regenerate tissue vascular grafts [315]. More recently, several multipotent adult stem cells including bone marrow cells (BMC), human mononuclear cells (MNCs) derived from bone marrow or peripheral blood, hematopoietic stem cells (HSCs) endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) with unlimited self renewal capacity and multilineage differential potential have been identified within the adult animal/humans [316]. Mounting evidences have shown that these multipotent adult stem cells circulate within the peripheral blood and migrate to injured tissues and contribute to tissue repair process *in vivo* [317, 318].These multipotent stem cells can be seeded on to the scaffold to generate the small diameter tissue engineered vascular grafts (TEVG) [315] *in vivo* which can be used as an alternative for autografts in bypass surgery (Table 1-2).

Table 1-2. Potential therapeutic applications of adult multipotent stem cells for the generation of small diameter tissue engineered vascular grafts (TEVG) *in vivo.*

Sno	Cell source	Scaffold	Animal model	Result
1	Muscle-derived stem cells (MDSCs) [319],	Poly(ester urethane)urea scaffolds	Rat	A smooth-muscle-like layer of cells was observed
2	Endothelial progenitor cells [320]	heparin-coated decellularized vessels (DV)	Dog	complete endothelium regeneration was seen
3	Bone marrow mononuclear cells (BM-MNCs) [321]	nonwoven polyglycolic acid mesh	Mouse	A luminal endothelial lining surrounded by concentric smooth muscle cell layer was found
4	Bone marrow-derived mononuclear cells (BMMCs) [322]	nonwoven polyglycolic acid (PGA) mesh	Mouse	A Endothelialized intimal layer and smooth muscle medial layer was observed
5	Bone marrow cell (BMC) [323, 324]	poly-l-lactic acid (PLLA)	Dog	A Endothelialized layer and smooth muscle medial layer was observed

1.15. Specific Aims

The aim of this study was to identify molecular mechanisms that control myocardin-dependent differentiation of SMSPCs into SMCs. The specific aims of this work are summarised below,

Aim 1: to characterise undifferentiated hSMSPC cells for the presence of smooth muscle transcription factors (SRF and myocardin), smooth muscle fate regulating miRNAs (miRNA 143 and miRNA145), smooth muscle markers (SM α actin, calponin and SMMHC) and smooth muscle repressors (KLF4, KLF5 and Elk-1) using qRT-PCR assay. This work also aims to identify key culture conditions and cytokines that can induce hSMSPC differentiation into SMCs

Aim 2: to identify the transcriptional repressor involved in smooth muscle differentiation of hSMSPC.

- This work aims to explore the putative binding sites for smooth muscle transcriptional repressors within the human myocardin promoter region.
- More specifically aims to investigate whether knockdown of KLF4 can induce the expression of myocardin promoter activity and to verify the KLF4 putative binding site on human myocardin promoter region using Chip assay.

Aim 3: to determine the molecular mechanism underlying myocardin mediated smooth muscle differentiation in hSMSPCs.

- Specifically, the first part aims to investigate whether TGF-β1 stimulation can induce SMC differentiation of KLF4 knockdown hSMSPC cells *in vitro.*
- The second part of this study aims to determine whether overexpression of myocardin cDNA can induce SMC differentiation of hSMSPCs

Aim 4: to determine the tissue capsule formation potential of rSMSPC eGFP cells using a rat model. In this work the integration of rSMSPC eGFP cells within the peritoneal derived tissue capsule in rat model will be assessed.

2. General methods and materials

2.1. Cell Culture

Cells were grown in an appropriate media as listed in Table 2-1 and maintained typically in 5% $\underline{CO_2}$ at 37°C in a cell incubator. Confluent adherent cells were sub cultured by monitoring the red to yellow pH indicator in media. The monolayer cells were washed twice with dPBS (Sigma, St.Louis, MO) in order to remove the serum present in the media and incubated with 0.25% trypsin (Sigma, St.Louis, MO) to release the adherent cells from the flask. The trypsin in detached cell suspension was neutralised by adding fresh serum containing media and sub cultured into new tissue cultured flasks.

2.2. Cell types

Table 2-1. List of *in vitro* cell types

Cell type	Description	Media	Reference
HeK 293T	Human Embryonic Kidney 293 cell variant expressing SV40 Large T antigen.	Dulbecco's Modified Media (DMEM) with 10% Fetal Bovine Serum (FBS)	ATCC, University Manassas, USA.
HUVEC	Human Umbilical Vein Endothelial Cells	EGM2 (Endothelial Cell Growth Media 2)	Lonza, Basel, Switzerland.
HASMC	Human Aortic Smooth Muscle Cells	SmGM2 (Smooth Muscle Growth Medium 2)	Cascade Biologics, Eugene, USA
rSMC	Rat Smooth Muscle Cells	DMEM with 10% FBS	ATCC, University Manassas, USA
hSMSPC	Human Smooth Muscle Stem Progenitor Cell	EGM2	Isolated from peripheral blood
rSMSPC eGFP	Rat Smooth Muscle Stem Progenitor Cell expressing eGFP	EGM2	Isolated from rat bone marrow

2.3. Antibodies

Table 2-2. List of antibodies used for immunofluorescence staining and western blotting.

Antibody	Clone	Company	Cat.log	Dilution
SM α actin	mIgG 2a 1A4	Dako	M0851	1 in 200
Calponin	Rbt IgG EP798Y	Epitomics	1806-1	1 in 200
SMMHC	Rat IgG 2a KM 3669	Kamiya biomedical company	MC-352	1 in 200
HA-tag	Rbt IgG	Sigma	H6908	1 in 100
GKLF	Rbt IgG	Santacruz	SC-20691	1in 200
Myocardin	mIgG	Abcam	Ab 22621	1 in 100
PCNA	mIgG	Chemicon Temecula, CA	CBL 407	1 in 1000
Rat IgG 2a		eBiosciences	13-4321-82	
Rabbit IgG		Prosci Incorporated	3703	
Mouse IgG 2a		Santacruz	SC-3878	
Goat anti mouse Alexafluor 546		Invitrogen	A11030	1 in 500
Goat anti rabbit Alexafluor 488		Invitrogen	A11008	1 in 500
Goat anti rat Alexafluor 488		Invitrogen	A11006	1 in 500

2.4. Quantitative reverse transcriptase *PCR* (qRT-PCR) primers

Table 2-3. List of qRT-PCR primers.

Marker	Gene Symbol Forward/Reverse	Sequence 5'->-3'
hMyocardin	hMYOCD_F	tcagcaatttcagaggtaacaca
	hMYOCD _R	tgactccgggtcatttgc
hSRF	hSRF _L	agcacagacctcacgcaga
	hSRF _R	gttgtgggcacggatgac
hCalponin	hCNN1 _L	ccaaccatacacaggtgcag
	hCNN1 _R	tcaccttgtttcctttcgtctt
hSM α actin	hACTA2 _L	ctgttccagccatccttcat
	hACTA2 _R	tcatgatgctgttgtaggtggt
hSMMHC	hMYH11_L	aactcgtgtccaacctggaa
	hMYH11_R	ttctcctcggctaacaactga
hKLF4	KLF4_L	gccgctccattaccaaga
	KLF4_R	tcttcccctctttggcttg
hKLF5	hKLF 5_L	ctgcctccagaggacctg
	hKLF 5_R	tcgtctatacttttatgctctggaat
hElk-1	Elk1_L	tgcttcctacgcatacattga
	Elk1_R	ggtgctccagaagtgaatgc
hGAPDH	hGAPDH_L	agccacatcgctcagacac
	hGAPDH_R	gcccaatacgaccaaatcc

2.5. RNA isolation

Total cellular RNA was isolated from tissue culture cell using RNeasy RNA isolation kit (Qiagen, Hilden, Germany) and miRNA was isolated using mirVana miRNA isolation kit (Invitrogen, Carlsbad, CA) according to the manufacture's instructions.

2.6. QRT-PCR

2 μg of total RNA was reversed transcribed into cDNA using SuperScript III (Invitrogen, Carlsbad, CA). Real-time RT-PCR (qRT-PCR) was carried out with 100 ng of cDNA specific primer for each qRT-PCR reaction with primer specific probe

and SYBR green (Roche Applied Sciences, West Sussex, UK). The quantification was done relative to GAPDH expression in each sample. PCR amplification with no RNA, no RT and water only served as negative controls. Probe specific qRT-PCR primers and SYBR green qRT-PCR primers were designed using the Roche – universal probe library (www.roche-applied-science.com) and Primer 3 software respectively.

2.7. Immunofluorescence microscopy

The cells were washed with phosphate buffered saline (PBS) (Sigma, St.Louis, MO), fixed with chilled methanol (Sigma, St.Louis, MO) for 10 minutes at -20^{O}C and washed twice with PBS for 5 minutes each at room temperature (RT). The non specific binding sites were blocked by incubating the cells in blocking buffer (10% goat serum in 0.1% Triton X-100/ PBS (Sigma, St.Louis, MO)) for 20 minutes in humidified chamber at RT. The cells were labelled with primary monoclonal antibodies for 2 hours at RT and washed thrice with PBS to remove the excess primary antibody. The cells were stained with alexafluor conjugated secondary antibody as listed in table 2-2 for 45 minutes at RT and washed three times with PBS followed by 10 minutes staining of the nuclei with 10µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA) in sterile water. The slides were washed with PBS, dried and mounted with immu-Mount (Thermo Shandon Inc, Pittsburgh, PA). The slides were analysed using TE 2000 Nikon confocal microscope using EZ-C1 version 3.90 software.

2.8. Fluorescence-activated cell sorting (FACS) analysis

The media was removed from the cells, washed once with PBS and incubated in 0.25% trypsin for five minutes. The tissue culture flask was tapped gently and flushed with media to collect the cells and centrifuged at 600g for 5 minutes. The pellet was washed once with FACS buffer (0.5% BSA, 1 mM Ethylenediaminetetraacetic acid (EDTA) (Sigma, St.Louis, MO) in PBS) and resuspended in FACS buffer. The FACS analysis was carried out in BD FACS calibur (BD Biosciences, San Jose, USA) cytometer. The live cells were gated on forward and side scatter and eGFP signal was collected in the FL1 channel. The FACS data was analysed for percentage eGFP expression by 2D dot plot using WinMDI (Windows Multiple Document **I**nterface for flow cytometry) 9.2 software.

2.9. Lentiviral production

Lentivirus was generated in HeK 293T cells. A day before transfection, 16 x 10^6 cells were seeded onto 15 cm tissue culture dish in DMEM supplemented with 10% FBS. The media was changed four hours before transfection to induce the growth of the cells. The HeK 293T cells were transfected with three plasmids (30 μg transfer construct, 24 μg packaging construct pCMV-ΔR8.91 encoding packaging protein Gag-Pol, Rev, Tat and 16 μg envelope pMD.G – encoding G protein of the vesicular stomatitis virus (VSV)) using standard calcium phosphate precipitation method as described below. The three plasmids were mixed together and the volume was made to 1.8 ml with sterile water and added 200 µl of $CaCl_2$. 2 ml of *N, N*-bis [2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) (Calbiochem, Middlesex, UK), pH 7.0 was added to the DNA/$CaCl_2$ complex in a drop wise manner while vortexing and incubated at room temperature for 5 minutes. The transfection mix was added to the cells in a drop wise manner and incubated in 3% CO_2 at 37^{O}C. The media was replaced 16 hours post transfection with DMEM supplemented with 2% FBS and incubated in 5% CO_2 at 37^{O}C. Lentivirus was harvested 48 hours after withdrawal of transfection media. The viral supernatant was collected and centrifuged at 600 g for 5 minutes at 4^{O}C and filtered through 0.45 µm filter. The lentivirus was concentrated by ultrafiltration using centricon-70 (Millipore, Billerica, MA) which remove residual VSV-G and pelleted by ultracentrifugation in a Sorval WX ultra series centrifuge (Thermo electro corporation, Waltham, Massachusetts) with swing rotor at 72,000 g for 90 min at 4^{O}C and resuspended in fresh media. Using these protocols, titers of ~5 x 10^7 to 3 x 10^8 TU/mL were achieved. The virus titre was estimated based on the transduction of HeK 293 T cells. A day before transduction HeK 293T cells were seeded at plating density 5 x 10^4 cells per well in a 24 well plate with DMEM media. Next day the media was removed and the cells were transduced with serial dilution of the concentrated vector that corresponds to the final amount of 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} µl of vector. After 72 hours of transduction, the cells were analysed for eGFP expression or the cells were stained with HA-tag antibody and percentage of HA-tag positive cells were determined using FACS analysis. The lentivirus titre was calculated using the following formula

$$\text{Titre (Transducing units/ml)} = \frac{\text{Number of target cells x (Percentage of eGFP}^+\text{ cells/100)}}{\text{Volume of vector (ml)}}$$

2.10. ShKLF 4 Lentivirus production protocol

A day before the transfection HeK 293T cells were seeded at 50,000 cells per cm^2 plating density in 6 well plate in DMEM 10% media. Next day the cells were transfected using 3:1 ratio of X-tremeGENE HP DNA transfection reagent (µl) (Roche Applied Sciences, West Sussex, UK) and plasmid DNA (µg). First the plasmid DNA mix comprised of three plasmids, 0.5µg of transfer construct shKLF4 Nature B, 0.25µg packaging construct pCMV-∆R8.91 and 0.25µg envelope pMD.G was diluted in diluent Opti-MEM I (Invitrogen, Carlsbad, CA) and than 3µl of X-tremeGENE HP DNA transfection reagent was added to the diluted plasmid DNA to form transfection complex. The transfection reagent: DNA complex was mixed and incubated for 20 minutes in the dark at room temperature. The transfection complex was added to the cells in a drop wise manner without removing the media. After 16 hours of transfection, the media was changed to DMEM supplemented with 2% FBS. Lentivirus was harvested 48 hours after removal of transfection media. The viral supernatant was collected and centrifuged at 600 g for 5 minutes at 4^OC and filtered through 0.45 µm filter and stored at -70^OC. Since the culture vessel size is larger 15 cm (176 cm^2) for the production of lentiviral constructs the amount of DNA used was higher (70 µg) as compared to shRNA lentivirus production in 6 well plate (9.6 cm^2) with 5 µg of DNA.

2.11. Transduction Protocol

Cells were maintained in media outlined in table 2-1. One day before transduction, the cells were seeded at 50,000 cells per cm^2 plating density in 100 µl EGM2 media in 8 well chamber slides. Next day, the cells were transduced with 5 MOI of lentivirus in 100 µl EGM2 media. After 24 hours of transduction, the growth media was changed to 200 µl either differentiation media (SmGM2 1%FBS with TGF β1 5ng/ml) or SmGM2 1%FBS. Fresh media (SmGm2 1% FBS) of volume 100µl was added every 3 days without removing the conditioned media.

2.12. Transient transfection

The hSMSPC cells were co-transfected by using lipofectamine (Invitrogen, Carlsbad, CA) with reporter plasmids encoding firefly (*Photinus pyralis*) luciferase pGL3 Enhancer-hMyocd driven by human myocardin promoter and renilla (*Renilla reniformis*) luciferase construct pRL-TK driven by thymidylate kinase- a housekeeping gene promoter in 25:1 ratio. The reporter plasmids were mixed gently in Opti-MEM I reduced serum media (Invitrogen, Carlsbad, CA) and

lipofectamine was diluted in Opti-MEM I. The diluted reporter plasmids was combined with diluted lipofectamine and incubated at room temperature for 45 minutes. After incubation, the volume was made up to 50 µl with Opti-MEM I and mixed gently. The growth media from cells were removed and replaced with 150 µl of EGM2. 50 µl of transfection complex were added to the each well in drop wise manner. The cells were incubated in 5% CO_2 at 37^{O}C.

2.13. Dual Luciferase Assay

In a dual luciferase assay the firefly and renilla luciferase activity were measured sequentially from a single sample. The glomax 20/20 luminometer (Promega, Wisconsin, USA) was programmed for Dual injection with a 10 second integration time. 100 µl of Firefly assay buffer (25mM Glycylglycine, 15mM K_2PO_4 pH8, 4mM ethylene glycol tetraacetic acid (EGTA), 15mM MgSO4, 0.1mM Coenzyme A, 75µM Luciferin , 2mM ATP (Sigma, St. Louis, MO) and 1mM Dithiothreitol (DTT) (Invitrogen, Carlsbad, CA)) was added to 20µl of cell lysate. The tube was placed in the luminometer and initiated the luminescence activity. The luminescence activity was measured for 10 seconds and referred as relative luminescence units (RLU) RLUs1. After 10 seconds, 100) µl of renilla luciferase buffer (1.1M NaCl, 2.2mM EDTA, 220 mM K_2PO_4 pH5.1, 0.44 mg/ml BSA, 1.3mM NaN_3, and 1.43 µM Coelenterazine h (Sigma, St. Louis, MO)) was added and luminescence activity was measured for 10 sec and referred as RLUs2. The RLUs1/RLUs2 ratio was calculated for each sample.

2.14. Western Blot analysis

The cells were washed and scraped in ice cold PBS, centrifuged for 12000 rpm at 4^{O}C for 10 minutes. The pellet was resuspended in 60 µ1 of crack lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS and 10% glycerol supplemented with Complete protease inhibitor (Roche Applied Sciences, West Sussex, UK) and phosphatase inhibitor (Sigma, St. Louis, MO). The lysate was sheared by passing five times through 23-gauge needle and boiled (99^{O}C) for 10 minutes, centrifuged for 14,000g at 4^{O}C for 10 minutes. The supernatant was collected and stored at -70^{O}C. The protein concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

The BCA assay reagent A and B was mixed at 50:1 ratio to obtain a bright green colour solution and added 200µl to each well in 96 well plate. 10 µl of cell lysate or known concentrations of reference protein (BSA) standards were added to

BCA assay mixture.The plate was incubated for 30 minutes at 37^{o}C and the colour change from green to purple color was measured at 540 nm using a plate reader. A standard curve was plotted using absorbance and known concentration of reference protein (BSA). The concentration of unknown sample was calculated using standard curve and used 50µg of protein sample for western blot analysis.

Protein was denatured and loaded onto an SDS-polyacrylamide gel with 4% stacking and 12% separating gels (1.5mm thick) and run at 80-120 V and constant current. 50 µg of protein from each sample were mixed with an appropriate volume of loading buffer to give a final volume of 30 µl for loading. The samples were boiled for 5 min and then loaded on the stacking gel along with a prestained protein marker (broad range 6-175 kDa, New England Biolabs, Lpswich, MA) to determine the molecular mass of proteins. Protein was transferred onto nitrocellulose membrane (GE Healthcare, Littlechalfont, UK). Prior to protein transfer, the membrane was soaked in dH2O and pre-chilled blot buffer. Transfer was carried out at 85V constant voltage for 1 hour. Transfer of protein was routinely checked by Ponceau S staining (Thermo Fisher Scientific, Waltham, MA). After transfer of proteins, the membrane was blocked (shaking) in blocking buffer (5% milk in TBST (50mM Tris HCl pH 8.0, 0.15M NaCl, 0.05% Tween) buffer for 30 min at room temperature. The membrane was incubated with the primary antibody diluted in 5% milk in TBST buffer. To remove excess of primary antibody, the membrane was washed with TBST, three times for 10 min. Next, the membrane was incubated for one hour at RT with the horseradish peroxidase (HRP) labeled secondary antibody in TBST 5% milk solution. Blots were then washed with TBST three times for 10 min and finally with enhanced chemiluminescence (ECL) solution (Millipore, Massachusetts, USA) for 1 minute. The signal was recorded with MultiSpectral Imaging System (BioSpectrum®) camera. Bands quantified using Image J (NIH software).

2.15. Chromatin immunoprecipitation (ChIP) Assay

hSMSPCs (8 x 10^{6}) were cultured in EGM2 media on 10 cm tissue culture flask were scraped in ice cold PBS with 100 mM phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride (PMSF), centrifuged (600g, 5 min) and resuspended in 10 ml of EGM2 media. The harvested cells were chromatin immunoprecipitated as described by the manufacturer Active Motif (Active motif, Carlsbad, CA). The cells were fixed with 270 µl of 37% formaldehyde and then incubated at 37^{O}C for 10 minutes on a rocker and quenched with glycine for 5 minutes, centrifuged and resuspended in lysis buffer with protease inhibitor. The cells were sheared by

sonication, centrifuged and supernatant was collected. The size of the sonicated DNA showed a smear between 0.5 to 1 Kb as determined by 1% agarose gel electrophoresis. The immunoprecipitation was performed in total volume of 200 µl contained 25 µl of Protein G beads, 20 µl of ChIP buffer I, 100 µl of sheared chromatin, 1 µl of PIC, 2 µg of anti-KLF4 or 2 µg normal IgG and volume made to 200 µl with sterile water. The components were resuspended thoroughly and immunoprecipitation was performed over night at $4^{O}C$ on end to end rotator. The Protein G beads only and rabbit IgG were used as negative controls. The beads were washed three times with wash buffer and the chromatin was eluted, reversed the cross links at room temperature. The protein- chromatin complexes along with the INPUT sample taken before sonication was heated and subjected to proteinase K treatment. The INPUT DNA was diluted 10 times before PCR amplification. Purified DNA from chip assay or input DNA were used as template for PCR amplification with 10 µM forward and reverse primer and 2X LightCycler 480 SYBR green I master mix in 10µl reaction and subjected to 35 cycles using Roche LightCycler 480 system. Water and without template were used as PCR negative controls.

2.16. Hematoxylin and Eosin (H&E) Staining Protocol

The frozen sections were allowed to thaw briefly at room temperature and fixed with 4% paraformaldehyde for 10 minutes at room temperature. The slides were washed once with PBS for 5 minutes. The slides were immersed in hematoxylin solution for 5 minutes and dipped in 1% acid alcohol for 10 seconds than sections were stained with eosin for one minute, dried and mounted under a cover slip with DPX mounting media.

2.17. Tissue Engineering

Irradiated 10 mm lengths of 3 mm diameter silastic tubing are coated with fibronectin ($5\mu g/cm^2$) for 45 minutes at room temperature in a rocker. The silastic tubes along with 50 million rSMSPC eGFP cells in 3 ml of saline were injected into peritoneal cavity of hPAP fisher rats. The uncoated and fibronectin coated tubes were used as negative controls. The implants were removed after two weeks and examined for tube capsule formation. The capsule was removed from the tubes, OCT embedded, sectioned and subjected to H&E staining, eGFP detection and immunohistochemical analysis of smooth muscle marker protein expression.

2.18. Statistical analysis

Statistical analyses were performed using graphpad prism software. Statistical significance was tested by unpaired students t test and $p<0.05$ was considered statistically significant.

3. Specific Aim 1 - Characterisation of undifferentiated hSMSPC cells for the presence of smooth muscle associated transcription factors

3.1. Introduction to progenitor cells differentiation into SMC

Smooth muscle differentiation plays an important role in the pathogenesis of vascular diseases, which underlies the importance of understanding the molecular mechanism of smooth muscle differentiation. Stem cells have the ability to differentiate into many cell types including smooth muscle cells at the site of vascular injury in the tissue repair process [325]. Many investigators have identified several sources of the committed smooth muscle progenitor cells in bone marrow, peripheral blood, brain and liver [294]. Mounting evidence suggests that cytokines such as TGF- β1, PDGF-BB and hormone like growth factor retinoic acid influence progenitor cell differentiation into smooth muscle cells. Cytokines and growth factor involved in progenitor cell differentiation into SMCs are listed in the table below.

Table 3-1. List of progenitor cell source growth factor and cytokines involved in differentiation into SMC

Cell type	Cytokine /Growth factor	Dosage	Phenotype change
Embryonal carcinoma cell line (p19)	Retinoic acid	10^6 mol/L	Differentiated into fibroblast like cells expressing SM α actin [246].
A404		1 mM/L	Up to 90% cells differentiated in to SMC like cells expressing SM α actin, calponin, SM1 isoform of SMMHC [246].
ESC		10 mM	> 93% cells differentiated into SMC cells expressing SM α actin and SMMHC [249].
ESC $Sca1^+$	PDGF-BB	10 ng/ml	Differentiated into SMC cells expressing SM α actin, calponin and SMMHC [251].
Adventitial progenitor cells		10 ng/ml	Differentiated into SMC [250].
Neural crest cell line (Monc-1)	TGF-β1	5 ng/ml	Differentiated into SMC expressing SM α actin, SM 22α, calponin, SMγ actin, smoothelin and SMMHC and showed contraction muscarinic agonist carbachol [260].
Multipotent adult progenitor cells (MAPC)		2.5 ng/ml	Differentiated into SMC phenotype [326].
hESC – CD 34^+ subpopulation	PDGF-BB and TGF-β1	5 ng/ml and 2.5 ng/ml	Differentiated into SMC expressing SM α actin, SM 22α, calponin [327].

Extracellular matrix (ECM) plays a vital role in control of SMC differentiation. ECM contains agrin, fibulins and fibronectin which interacts with integrins and activate multiple signalling pathways [328]. Glycosylation of basement membrane enhances its binding of growth factors [329] and changes in ECM composition determine the phenotype of smooth muscle cell [327]. In the normal vessel media, dense ECM surrounding SMC helps to maintain a non proliferative SMC state [330]. Multipotent human $Isl1^+$ heart progenitor cells, when seeded on fibronectin coated culture plates differentiate into SMC [331]. Lu *et al.*, [332] demonstrated that hemangioblasts from ESC can be induced to differentiate into SMC expressing SM α actin, SM 22α and calponin when cultured on fibronectin coated slides in SMC media. In addition, CD 34+ subpopulations of hESCs were induced to SMC expressing smooth muscle markers such as SM α actin, SM 22 α and calponin. The cells were maintained on fibronectin coated culture surface in DMEM 5% FBS with PDGF-BB (5 ng/ml) and 2.5 ng/ml of TGF-β1 [327]. Taken together, these results suggest that the cytokine along with fibronectin can induce differentiation of progenitor cells into SMC.

In our lab, a novel $Isl\text{-}1^+$ stem progenitor cells was isolated from the peripheral circulation of human subjects. Interestingly, i*n vitro* characterisation of hSMSPC cells has provided first time evidence that these cells express higher levels of KLF4 as measured by FACS analysis, a possible candidate for regulating SMC differentiation in hSMSPC cells. It has been shown that the progenitor phenotype is maintained by expression of one or more repressors [302] and it is interesting to note that smooth muscle associated miR-143 and miR-145 are involved in downregulation of smooth muscle repressors, KLF4 and Elk-1 [208]. However, smooth muscle specific markers and miRNAs have not been identified yet in hSMSPC cells.

Therefore, the current **hypothesis that hSMSPC express low levels of smooth muscle associated transcription factor and higher levels of repressor**.

The present study aims to characterise undifferentiated hSMSPC cells for the presence of smooth muscle transcription factors (SRF and myocardin), smooth muscle fate regulating miRNAs (miRNA 143 and miRNA145), smooth muscle markers (SM α actin, calponin and SMMHC) and smooth muscle repressors (KLF4, KLF5 and Elk-1) using qRT-PCR assay.

The second part of this study aims to identify key culture conditions and smooth muscle associated cytokines that can induce hSMSPC differentiation into SMCs. Additional aims determine the role of growth media such as EGM2, EGM2

with 1% FBS, SmGM2, SmGM2 with 1% FBS and cytokines such as TGF-β1 (2.5 ng and 5 ng/ml), PDGF-BB (5 and 10 ng/ml) and retinoic acid (10 and 20 µM) to induce SMC differentiation in hSMSPC cells.

3.2. Methods

3.2.1. Cell culture

hSMSPC *Isl1*$^{+}$ progenitor cells were isolated from bypass filter of atherosclerosis patients undergoing CABG surgery. The total cells from filters were collected by flushing the filters with PBS and RPMI medium. The passaged cells were maintained in EGM2 medium. HASMCs were obtained from Cascade Biologics, Eugene, USA and maintained in SmGM2 medium. The cells were maintained at 37^{O}C in a humidified atmosphere containing 5% CO_2. The cells were passaged in to 75 cm^2 culture dish for RNA isolation and onto nunc wells for immunohistochemical analysis. HASMC from early passage was used for RNA isolation and immunohistochemical analysis.

3.2.2. RNA isolation

hSMSPC and HASMC cell were washed twice with dPBS and washed with 0.25% trypsin to lift the attached cells. The cells were pelleted by centrifugation at 600g for 5 minutes. Total cellular RNA from hSMSPC and HASMC was isolated using RNeasy RNA isolation kit according to the manufacture's instruction.

3.2.3. QRT-PCR

QRT-PCR analysis was carried out as outlined in general method section 2.5 with specific primers. The primers used were outline in general methods table 2-3.

3.2.4. hSMSPC differentiation protocol

3.2.4.1. Coating of Nunc wells with fibronectin

Fibronectin was diluted in sterile PBS and the 8 well nunc slides were coated with fibronectin at 5 µg/cm^2 concentration in minimal volume. The coated slides were allowed to air dry for 45 minutes at room temperature under the laminar air flow chamber.

3.2.4.2. Treatment of hSMSPC cells with different culture media to induce SMC differentiation.

The aim of this experiment was to characterise SMC differentiation from hSMSPC under various culture conditions. Confluent hSMSPC was detached from the culture dish and seeded at 10,000 cells per cm^2 onto fibronectin coated 8 well-nunc slides in the presence of four different media such as EGM2, EGM2 1% FBS, SmGM2, SmGM2 1%FBS. The hSMSPC were maintained in above mentioned growth media for 7 days. Later on, the cells were washed with PBS and immunostained for smooth muscle marker proteins as outlined in general methods section 2.7 with smooth muscle marker antibodies such as SM α actin (1 in 200), calponin (1 in 200) and SMMHC (1 in 200). The images were obtained with 60X oil immersion lens using confocal microscopy. The percentage of smooth muscle markers positive cells were counted in 10 randomly chosen 60X fields from three separate experiments.

3.2.4.3. Comparison of various cytokines and growth factor for their role in hSMSPC differentiation into SMC

The purpose of this experiment was to investigate the role of cytokines in hSMSPC differentiation into SMC. hSMSPCs were seeded at 10,000 per cm^2 onto fibronectin coated 8 well nunc slides in basal EGM2 medium. Next day, the medium was replaced with SmGM2 1% FBS alone with cytokines such as TGF-β1 (2.5 and 5 ng/ml), PDGF-BB (5 and 10 ng/ml) and retinoic acid (10 and 20 μM) respectively. After 3 days, fresh media was added to each treatment with appropriate cytokines without removing the conditioned media. At Day 7, the growth media was removed and washed with PBS and cells were immunostained for smooth muscle marker proteins as outlined in general methods section 2.7 with antibodies such as SM α actin (1 in 200), Calponin (1 in 200) and SMMHC (1 in 200). The images were obtained with 60X oil immersion lens using confocal microscopy. The percentage of smooth muscle positive cells relative to total number of cells were counted in 10 randomly selected high power fields (n=3).

3.3. Results

3.3.1. Characterisation of hSMSPC using qRT-PCR analysis

The expression levels of smooth muscle marker genes such as SM α actin, calponin and SMSMHC and smooth muscle transcription factors SRF, myocardin, miR-143 and miR-145 was analysed by qRT-PCR assay. In addition, the expression

of smooth muscle repressors including KLF4, KLF5 and Elk-1 was also analysed in undifferentiated hSMSPC cells. The expression levels of these markers in hSMSPC cells were compared with HASMCs (Fig. 3.1).

The results demonstrate that the undifferentiated hSMSPC cells express low levels of myocardin (Fig.3.1B), miR-143 and miR-145 (Fig.3.1C&D). It was also found that these cells express modest levels of SM α actin (Fig.3.1E), low levels of calponin (Fig.3.1F) and nearly equivalent levels of SMMHC (Fig.3.1G) when compared to HASMCs. However, they contain SRF mRNA levels equal to HASMC cells. Notably, hSMSPCs also express higher levels of repressors of myocardin–SRF dependent transcription including KLF4, KLF5 and Elk-1 (Fig. 3.1H-J). These repressors can inhibit myocardin induced SMC transcription [138, 167, 287], suggesting that the progenitor phenotype of hSMSPC cells may be maintained in an undifferentiated state by repression of myocardin gene expression by repressors.

3.3.2. Serum reduced smooth muscle medium (SmGM2 1%FBS) enhances differentiation of hSMSPC to Smooth muscle like cells.

hSMSPCs were maintained in EGM2, endothelial medium after isolation from the filters. To identify suitable media for hSMSPC differentiation these cells were grown in different growth media such as EGM2, serum reduced EGM2 (EGM2 1% FBS), SmGM2 and serum reduced SmGM2 (SmGM2 1%FBS) for 7 days (Fig.3.2.). Expression of smooth muscle marker proteins was detected by immunofluorescence analysis.

Undifferentiated hSMSPCs grown in basal EGM2 media showed a punctate staining pattern within the nucleus for SM α actin (Fig.3.2A). The reason for SM α actin punctuate is unclear. However, these cells did not express calponin (Fig.3.3A) and SMMHC at the protein level (Fig.3.4A). This result for SM α actin and calponin is consistent with the qRT-PCR analysis (Fig. 3.1E&F). Although, SMMHC (Fig.3.1 G) mRNA signal was detected by qRT-PCR, the SMMHC protein expression was not detectable by immunofluorescence analysis, suggesting that SMMHC gene expression levels are not high enough for translated protein expression or may be related to the presence of non-functional SMMHC protein in hSMSPC cells In order to optimise the growth media for hSMSPC differentiation, these cells were grown in various growth media. The cells maintained in smooth muscle growth media (SmGM2) and serum reduced SmGM2 1%FBS express SM α actin, to a greater extent (Fig.3.2 C&D). In these two treatments, the SM α actin expression is evenly distribution throughout the cells with 3.5 and 4 fold increase in SmGM2 and SmGM2 1% FBS (Fig. 3.2E), respectively**.** Furthermore**,** the

expression of calponin was increased to a similar extent in all treatments (Fig.3.3) as compared to basal medium (EGM2). However, there was no difference in SMMHC expression observed between different growth media (Fig.3.4). These results indicate that hSMSPC showed a potential to differentiate towards SM like cells when cultured in SmGM2 or SmGM2 supplemented with 1% FBS. Since the SmGM2 1% FBS showed more SM α actin positive cells, it was selected for further differentiation study.

3.3.3. TGF β1 induces differentiation of hSMSPC to SM like cells

Having shown that hSMSPC have the potential to differentiate into SMC in SmGM2 1%FBS, this study sought to test whether commonly associated smooth muscle cytokines can further enhance SMC differentiation of hSMSPC cells. Several studies of progenitor cells reported the role of TGF-β1, PDGF-BB and retinoic acid in SMC differentiation, survival and growth. In order to investigate the role of TGF-β1, PDGF-BB and retinoic acid in the hSMSPC differentiation process, two different concentrations of TGF-β1 (2.5 and 5 ng/ml), PDGF-BB (5 and 10 ng/ml) and retinoic acid (10 and 20 µM) were used in this study. hSMSPCs were suspended in EGM2 media and seeded at 10,000 cells/cm^2 density in the 8 well chamber slides. At day 1, the media was changed with SmGM2 1% FBS with 2.5, 5 ng/ml TGF-β1 or 5, 10 ng/ml PDGF-BB and 10, 20 µM retinoic acid respectively. At day 7, immunohistochemistry for SM markers was performed on all treatments along with untreated hSMSPCs.

Stimulation with PDGF-BB (5, 10 ng/ml) and retinoic acid (10, 20 µM) did not show any significant increase in the expression of SM α actin (Fig. 3.5), calponin (Fig. 3.6) and SMMHC (Fig. 3.7) at the protein level. However, TGF-β1 5 ng/ml showed a 5.7 fold increase in expression of SM α actin (Fig. 3.5) and only a slight increase in calponin expression was observed in hSMSPCs (Fig. 3.6). However, no difference in expression of SMMHC was induced by TGF-β1 treatment (Fig. 3.7). Taken together, these results suggest that TGF-β1 5 ng/ml signaling may be inducing SMC differentiation but expression of repressors in hSMSPCs may contribute to the inhibition of TGF-β1 induced SMC differentiation.

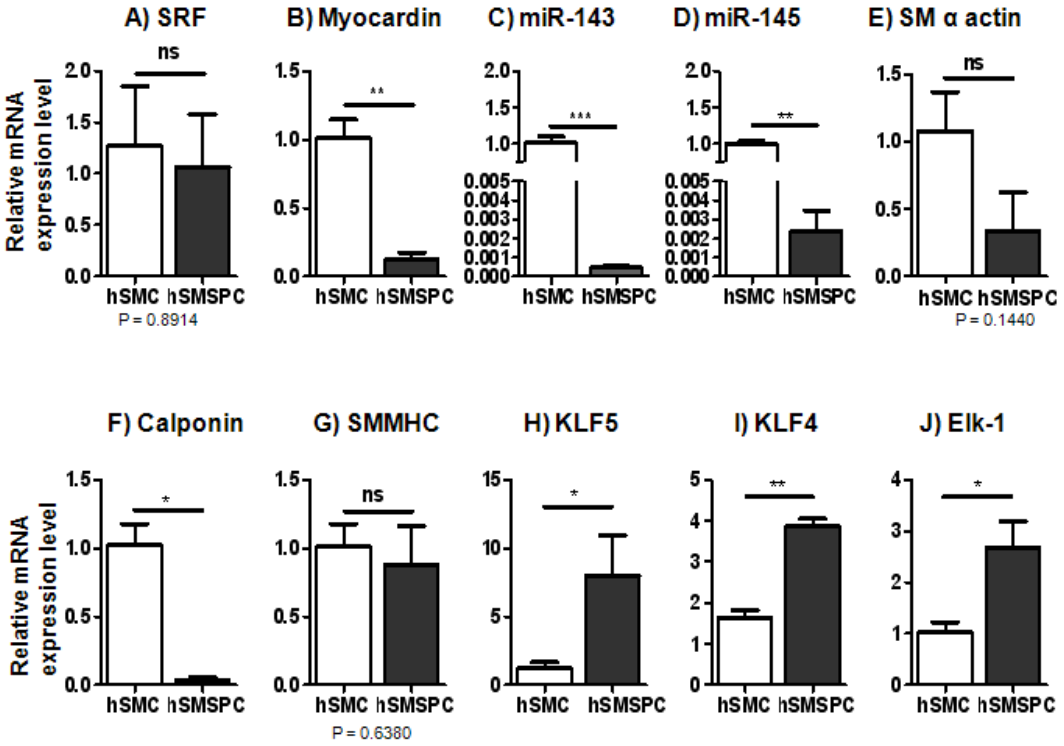


Figure 3-1. Characterisation of hSMSPC derived from human blood using qRT-PCR.

Total RNA was isolated from the undifferentiated hSMSPC grown in EGM2 media and hSMC grown in SmGM2 media. White bars indicate hSMC and black bars indicate hSMSPC. Comparative qRT-PCR analysis of smooth muscle transcription factors, SRF (A) and myocardin (B), smooth muscle cell fate regulating miRNAs, miR-143 (C) and miR-145 (D), smooth muscle markers SM α actin (E), calponin (F), SMMHC (G), and smooth muscle repressors KLF5 (H), KLF4 (I) and Elk-1 (J) between hSMSPC and hSMC. GAPDH was used to normalise the relative expression levels. ** P<0.01, *P<0.05, ns – not significant P>0.05

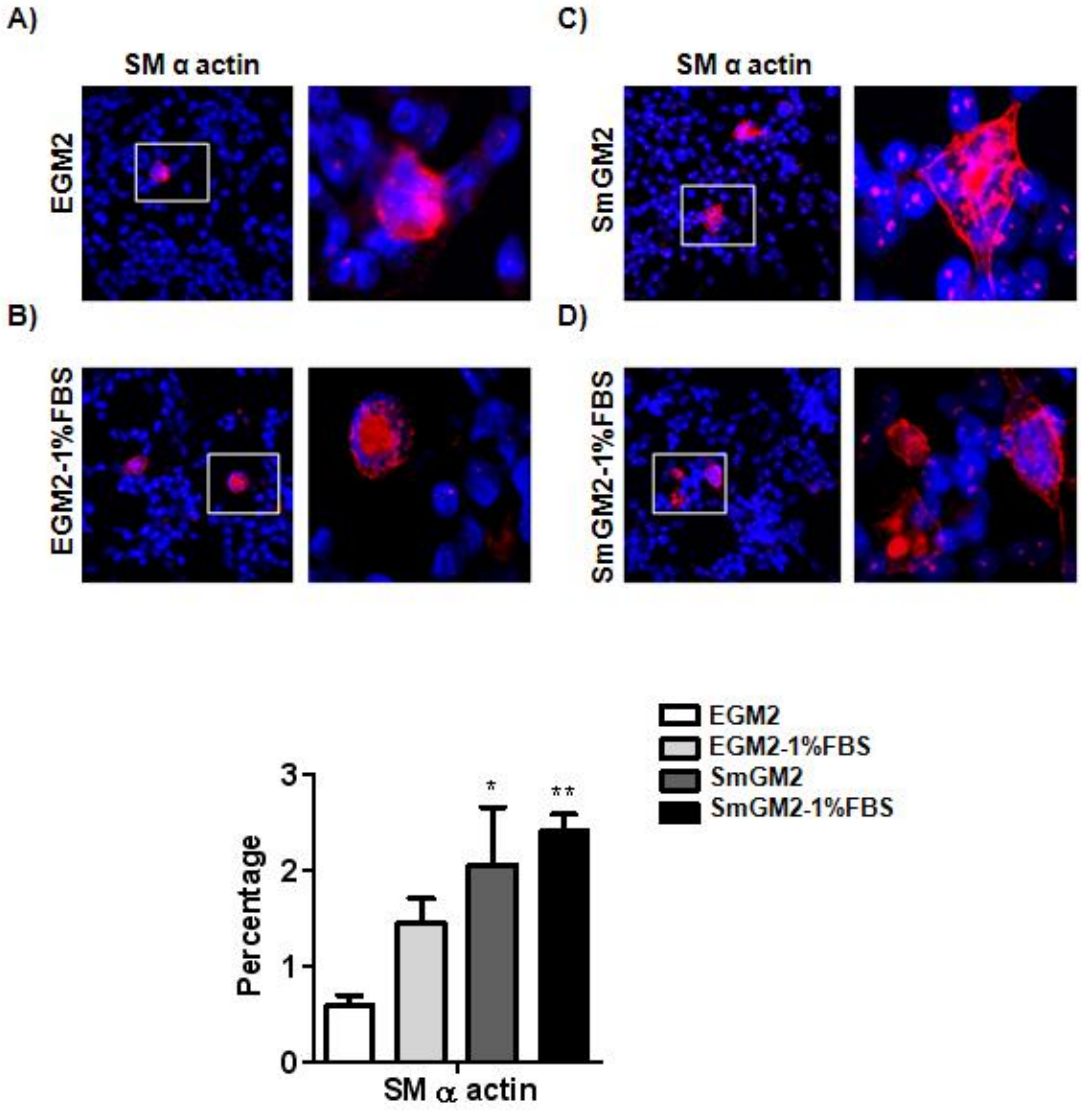


Figure 3-2. Growth media (SmGM2 1% FBS) induced differentiation in hSMSPC *in vitro*.

To optimise the media for hSMSPC differentiation, cells were plated at 2.5 x10^4 /cm^2 in EGM2, EGM2 1% FBS, SmGM2, and SmGM2 1% FBS growth media respectively on chamber slides for 7 days. The differentiated cells were immunostained with SM α actin antibody. The expression of SM α actin (red) in hSMSPC grown in EGM2 (A), EGM2 1%FBS (B), SmGM2 (C), and SmGM2 1% FBS (D) were shown Fig. 3.2. The white boxed area is enlarged and presented on right side of the each panel.The graph (E) represents the quantification of SM α actin positive cells in different growth media from n=3 independent experiments.

** P<0.01, *P<0.05

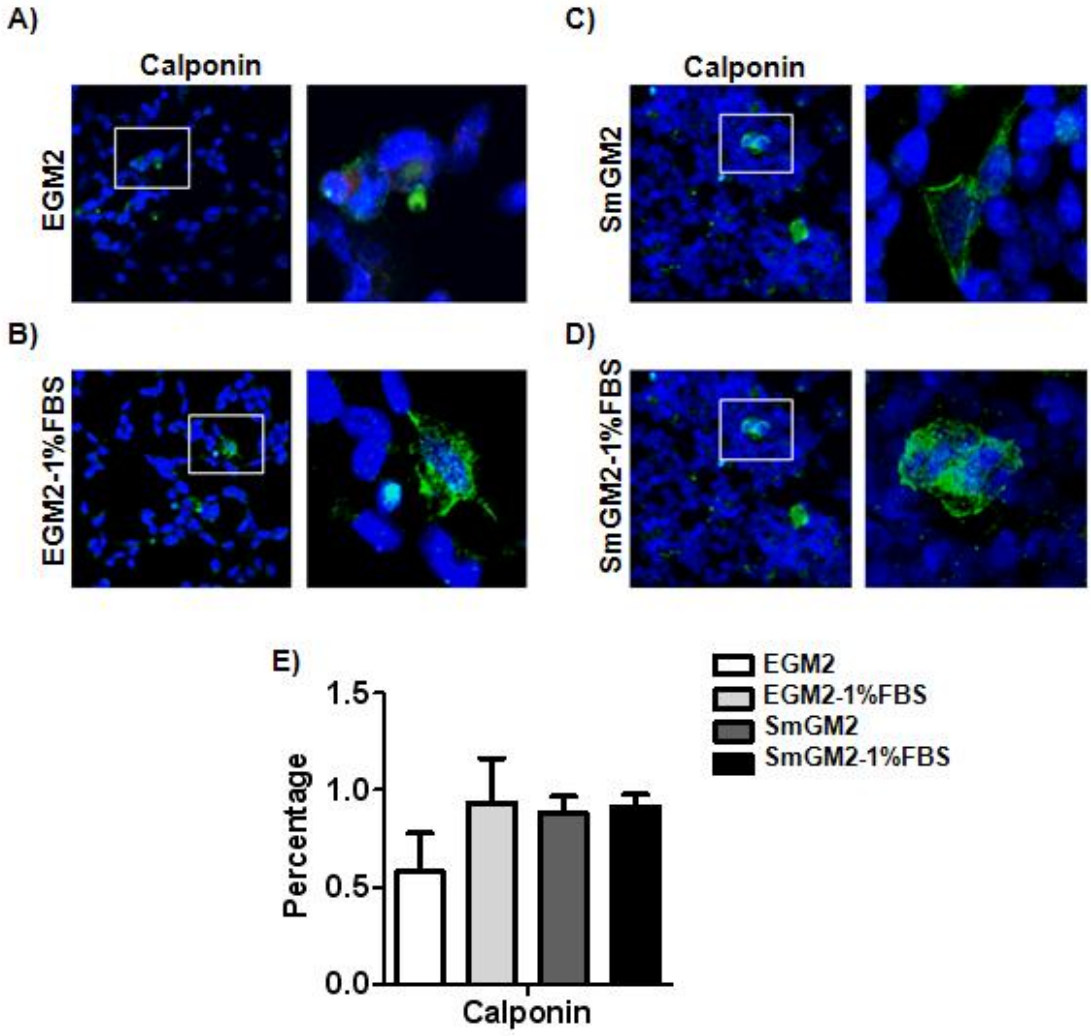


Figure 3-3. Growth media (SmGM2 1% FBS) induced differentiation in hSMSPC *in vitro*.

Cells were plated at 2.5 x10^4 /cm^2 in EGM2, EGM2 1% FBS, SmGM2, and SmGM2 1% FBS growth media respectively on chamber slides for 7 days. The differentiated cells were immunostained with calponin antibody. The differentiated hSMSPC cells were stained for calponin (green) and nucleus (blue) in EGM2 (A), EGM2 1%FBS (B), SmGM2 (C), and SmGM2 1% FBS (D) media and the small white rectangular boxed area shows the magnified field image in respective treatments. The graph (E) Represents the quantification of calponin positive cells in different growth media from n=3 independent experiments.

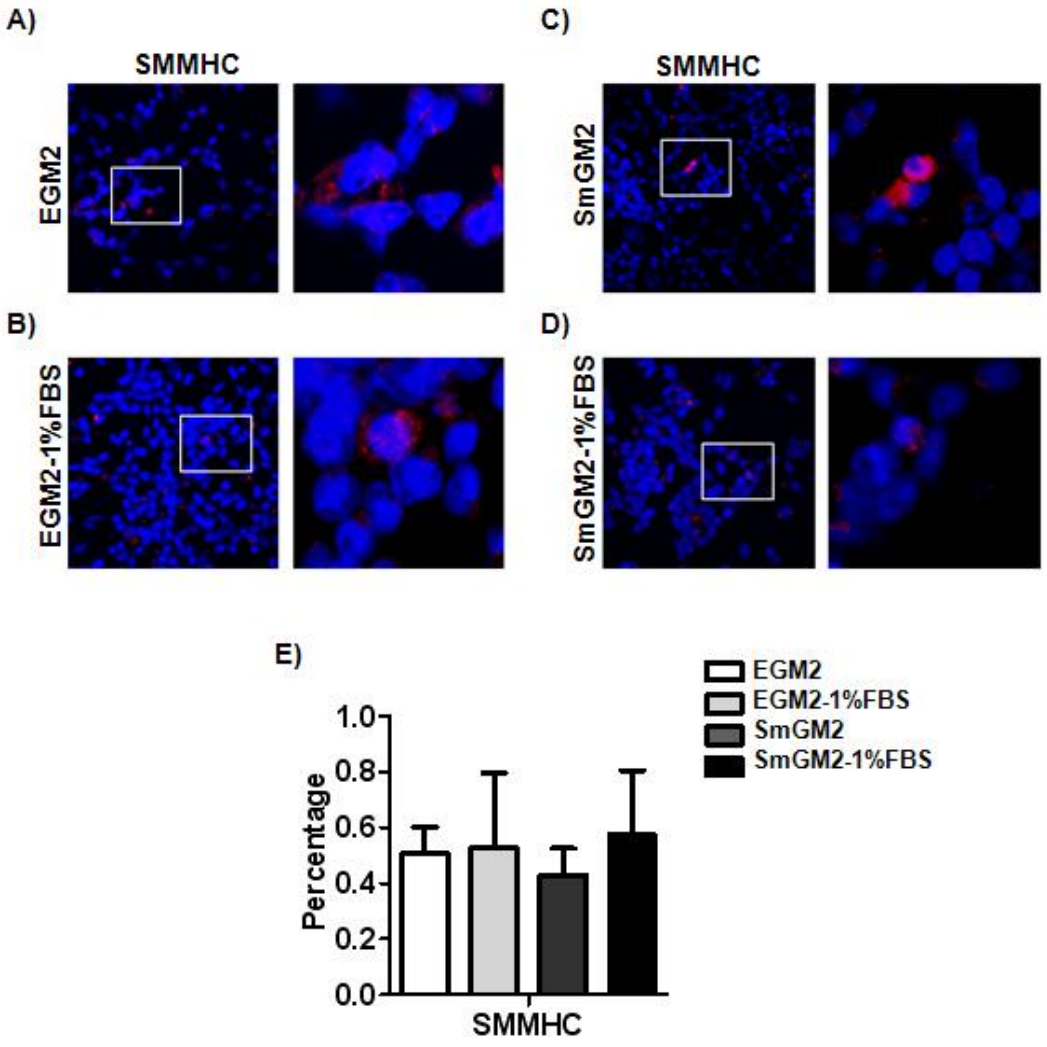


Figure 3-4. Growth media (SmGM2 1% FBS) induced differentiation in hSMSPC *in vitro.*

Cells were plated at 2.5 x10^4 /cm^2 in EGM2, EGM2 1% FBS, SmGM2, and SmGM2 1% FBS growth media respectively on nunc chamber slides for 7 days. The differentiated cells were immunostained with SMMHC antibody. The differentiated hSMSPC cells were stained for SMMHC (red) and nucleus (blue) in EGM2 (A), EGM2 1%FBS (B), SmGM2 (C), and SmGM2 1% FBS (D) media. A small portion marked as white small rectangular region was magnified and presented on to the left in each treatment. The graph (E) Represents the quantification of SMMHC positive cells in different growth media from n=3 independent experiments.

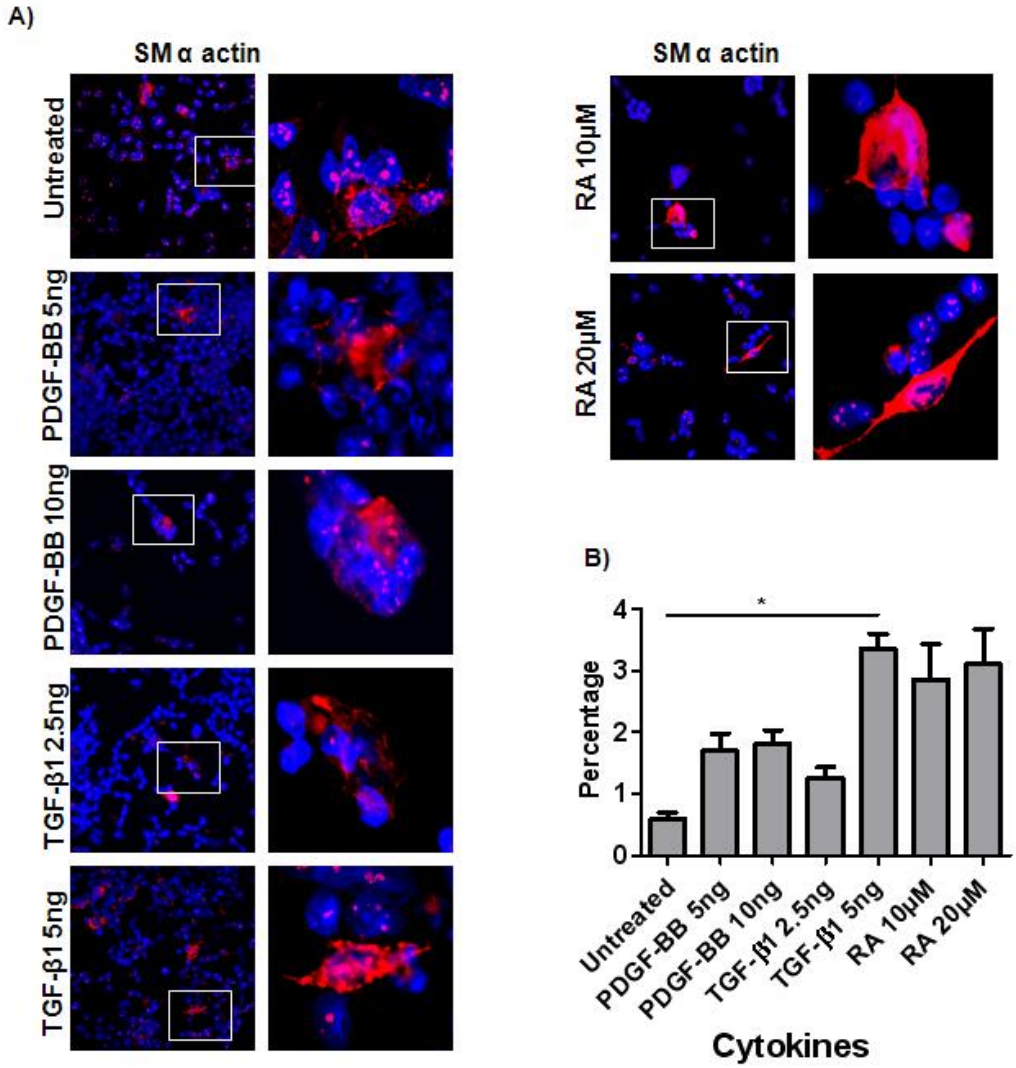


Figure 3-5. Cytokine-induced SMC differentiation (SM α actin) in hSMSPC.

hSMSPC cells were plated in EGM2 for 24 hours to facilitate attachment and the cells were differentiated in culture using various cytokines (TGF-β1 (2.5 and 5 ng/ml), PDGF-BB (5 and 10 ng/ml) and retinoic acid (RA) (10 and 20 μM)) in SmGM2 with 1% FBS media for 7 days. The differentiated cells were stained with SM α actin antibody for immunofluorescence analysis. Right panel shows differentiated hSMSPC cells stained for SM α actin (red) and nucleus (blue) in different cytokines and left panel shows the magnified field image in respective treatment. The graph (B) Represents the quantification of SM α actin positive cells in different cytokines from three different experiments. n=3, *P<0.05.

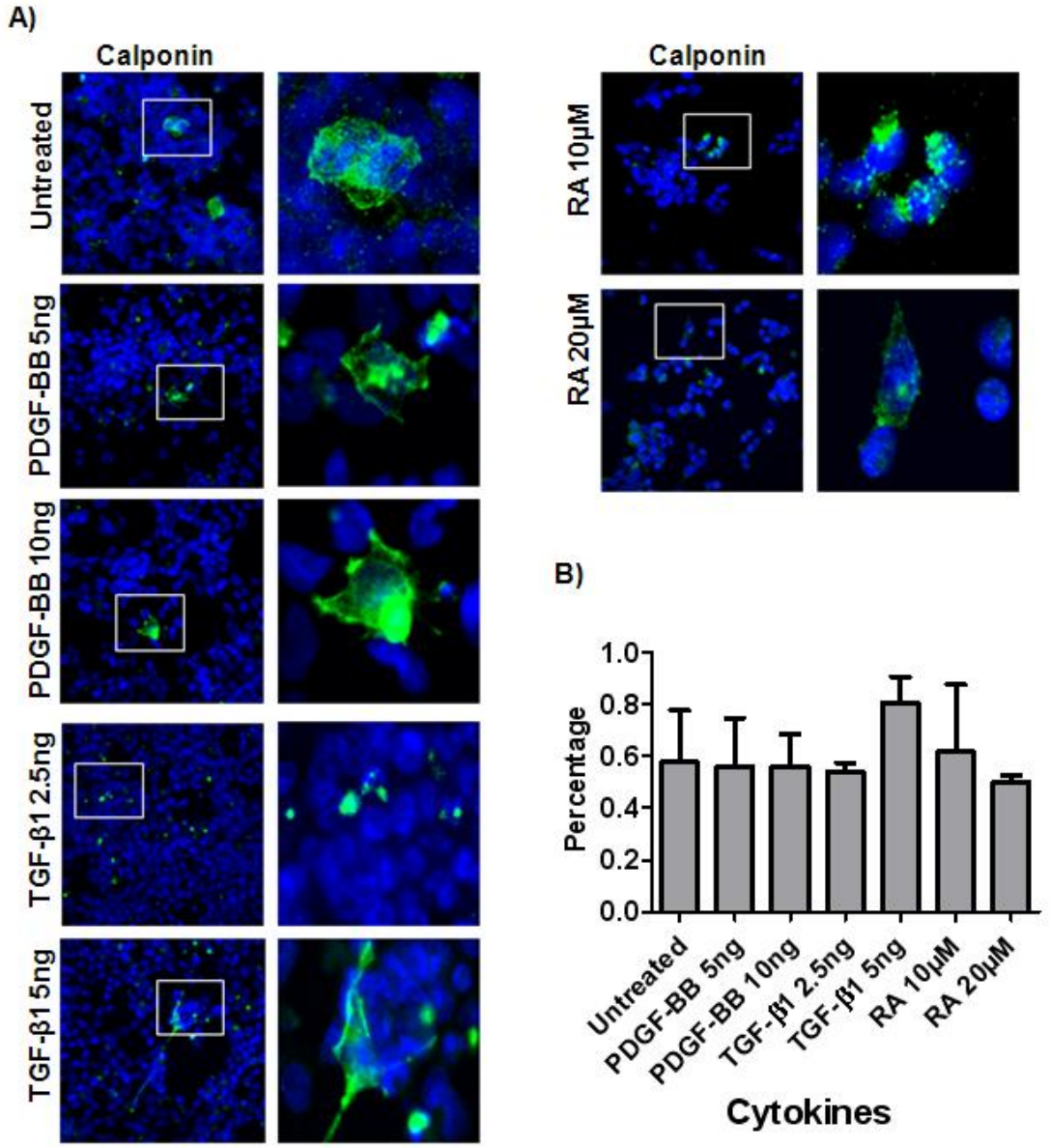


Figure 3-6. Cytokine-induced SMC differentiation (calponin) in hSMSPC.

hSMSPC cells were differentiated in culture using various cytokines (TGF-β1 (2.5 and 5 ng/ml), PDGF-BB (5 and 10 ng/ml) and retinoic acid (10 and 20 μM)) in SmGM2 with 1% FBS media for 7 days. The differentiated cells were stained with calponin antibody for immunofluorescence analysis. Right panel shows differentiated hSMSPC cells stained for calponin (green) and nucleus (blue) in different cytokines and left panel shows the magnified image in respective treatment inside the white rectangular region. The graph (B) represents the quantification of calponin positive cells in different cytokines from three different experiments. n=3.

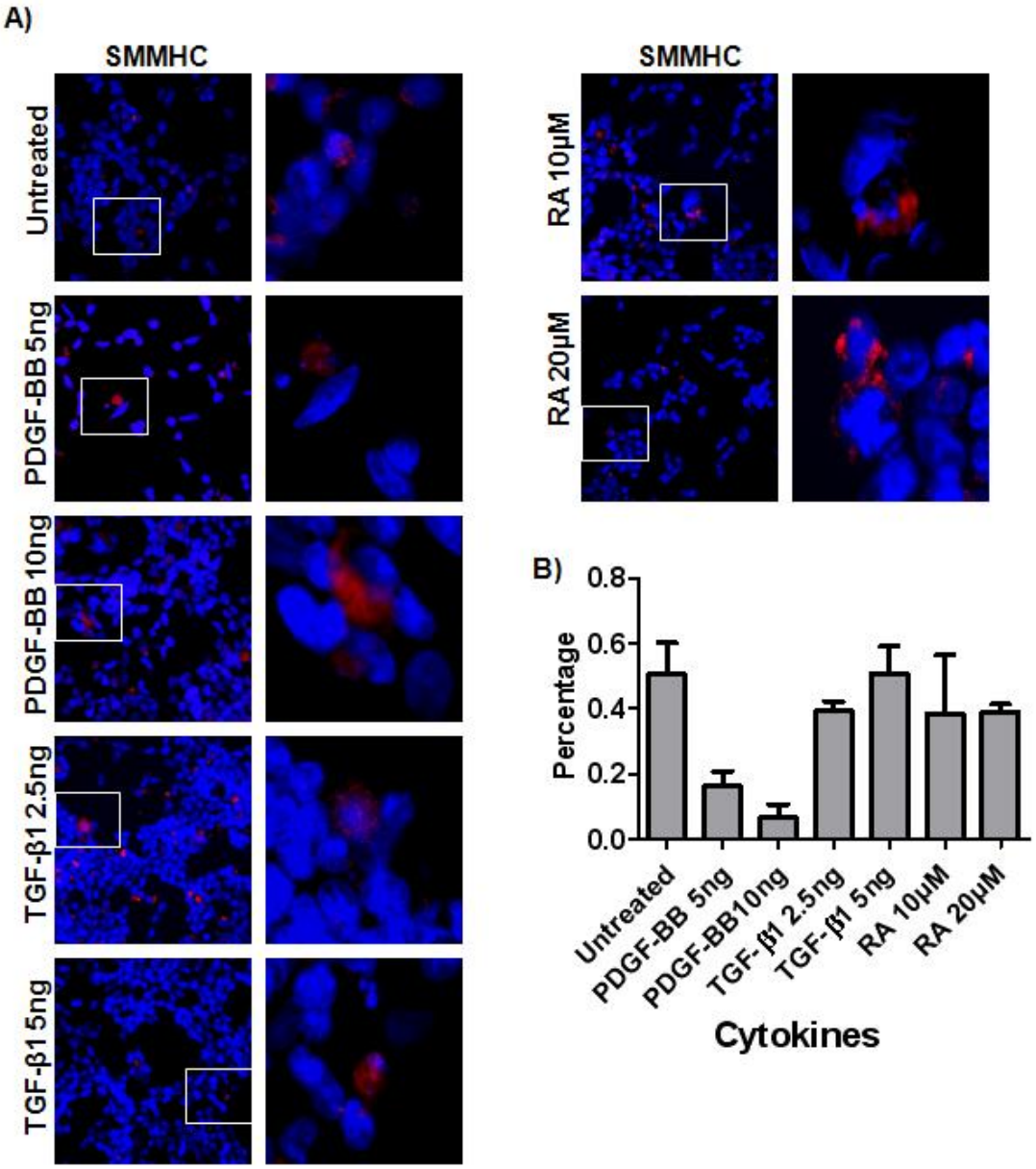


Figure 3-7. Cytokine-induced SMC differentiation (SMMHC) in hSMSPC.

hSMSPC cells were differentiated in culture using various cytokines (TGF-β1 (2.5 and 5 ng/ml), PDGF-BB (5 and 10 ng/ml) and retinoic acid (10 and 20 μM)) in SmGM2 with 1% FBS media for 7 days. The differentiated cells were stained with SMMHC antibody for immunofluorescence analysis. Right panel shows differentiated hSMSPC cells stained for SMMHC (red) and nucleus (blue) in different cytokines and left panel shows the magnified field image in respective treatment inside the white rectangular region. The graph (B) represents the quantification of SMMHC positive cells in different cytokines from three different experiments. n=3.

3.4. Discussion

The goal of these experiments was to identify the key transcription factors, growth media and cytokines that may regulate SMC differentiation in hSMSPCs. Results show that 1) hSMSPC cells do not express myocardin, smooth muscle markers, miR 143 and miR145 but they do express SRF, the basic SMC transcription factor. However, hSMSPCs express potent repressors for myocardin including KLF4 and Elk-1. 2) Serum reduced smooth muscle growth medium (SmGM2 1% FBS) facilitates smooth muscle differentiation in hSMSPCs 3) TGF-β1 stimulation in serum reduced SmGM2 (SmGM2 1% FBS) media induces hSMSPC differentiation towards smooth muscle like cells.

The major finding of this study comes from the qRT-PCR assay, which reveals that hSMSPC express smooth muscle transcription activators including myocardin and smooth muscle marker calponin at low level. In contrast, hSMSPC express smooth muscle repressors including KLF4, KLF5 and Elk-1 at higher levels. Recently, miR143/ 145 were shown to regulate smooth muscle cell fate in progenitor cells during development [208]. Notably, this study has identified low expression levels of miR 143/145 and higher expression levels of their target genes such as KLF4 and Elk-1 transcription. These results suggest that expression of these repressors may be responsible for suppression of myocardin induced SMC differentiation in hSMSPC cells.

Expression levels of smooth muscle markers including SM α actin and SMMHC were detectable by qRT-PCR assay. However, SM α actin was detected only as punctate staining within the nucleus using immunofluorescence analysis (Fig. 3.2A), suggesting the presence of non-functional splice variants in hSMSPC cells. SM α actin has in total seven transcripts, of which, four transcripts codes for functional proteins and three processed transcripts with no functional protein (Ensembl Gene ID: ACTA2 ENSG00000107796).

The SMMHC mRNA signal was detectable by qRT-PCR analysis but its protein level was not detected by immunofluorescence analysis, indicating the presence of non functional variants or that the mRNA levels are not sufficient to be converted into detectable protein in hSMSPC cells. SMMHC has 12 transcripts with six functional isoforms and six transcript variants (Ensembl Gene ID: MYH11 ENSG00000133392). However, the qRT-PCR primers for both SM α actin and SMMHC were designed in such a way to detect the full length functional isoforms along with their transcripts variants. These results indicate the presence of non-

functional SM α actin and SMMHC transcript variants in undifferentiated hSMSPC cells.

PDGF-BB [244, 245] and retinoic acid [246, 249] were known to induce smooth muscle differentiation of variety of cells. PDGF-BB acts as chemoattractant during embryonic development and mediates the recruitment of SMC precursor cells and pericytes towards endothelial cells [333]. However, after birth, PDGF-BB is associated with vascular injury induced SMC proliferation [105] and suppression of SMC differentiation proteins [334]**.** Expression levels of retinoic acid is involved in SMC development and maturation [335]**,** inhibition of SMC proliferation [336] and enhancement of the contractile SM proteins [337]**.** In addition, retinoic acid induces differentiation of p19 and ESC into SMC [246, 249].

When cultures of hSMSPC cells were treated with PDGF-BB and retinoic acid, they do not show any drastic changes in the smooth muscle marker expression. Moreover, retinoic acid treatment in hSMSPC cells resulted in detachment of cells from the surface of the culture plate. Notably, TGF-β1 treated hSMSPC cells were differentiated towards smooth like phenotype with increased expression of SM α actin and to a lesser extent calponin. TGF β1 is a potent cytokine involved in vascular smooth muscle cell differentiation [241]. However, TGF-β1 treatment in hSMSPC cells was unable to induce calponin and SMMHC to a greater extent, suggesting that TGF-β1-myocardin induced signaling may be controlled by expression of repressor KLF4 in hSMSPC cells.

Notably, KLF4 has been shown to bind the TCE regions of smooth muscle promoters and negatively regulate TGF-β1 induced smooth muscle gene transcription [139]. Furthermore, knockdown of smooth muscle repressor KLF4 enhanced the differentiation of ESC [338]. It would be interesting to test whether knockdown of KLF4 might enhance the TGF-β1 induced smooth muscle gene transcription in hSMSPC cells. KLF4 was also reported to ablate myocardin induced activation of CArG dependent smooth muscle genes through hypoacetylation of CArG elements [138, 167]. The presence of KLF4 may be a major contributing factor for downregulation of myocardin gene expression. Therefore, it will be important to evaluate the transcriptional regulation of myocardin gene expression by KLF4 in hSMSPC cells.

4. Specific Aim 2 – Identification of transcriptional repressor involved in smooth muscle differentiation in hSMSPC

4.1. Introduction

Smooth muscle gene expression varies between different stages of development and involves various regulatory elements and signaling pathways [75, 105]. Several cis regulatory elements (TCE element [139, 140], G/C repressors [99, 339] and E boxes [340]) have been identified within smooth muscle gene promoters and have been reported to be involved in the regulation of smooth muscle gene expression. The complexity in smooth muscle gene regulation is achieved by a unique combination of transcription factors binding to cis regulatory elements in smooth muscle promoter specific manner.

Myocardin is a transcription cofactor that physically interacts with SRF activating smooth muscle gene transactivation [142, 151]. Several repressor proteins including Elk-1 [127], Forkhead transcription factors 4 (Foxo 4) [301], KLF4 [138, 167], *HES*-related repressor protein 1 (HERP1) [341], High mobility group 2 like 1 protein (HMG2L1) [342], Thymine DNA glycosylate (TDG) [343], Runt related transcription factor 2 (Runx 2) [344], Carboxyl terminus of hsp70-interacting protein**)** ligase **(**CHIP) [166], Msx1 and Msx1 [165] have been reported to inhibit myocardin-induced SMC genes. Repressor proteins inhibit myocardin-induced SMC transcription through their interaction with myocardin basic and SAP domain [301], blocking the formation of SRF/myocardin complex [344], formation of a ternary complex with SRF and myocardin [165], decreasing myocardin stability via proteasome mediated degradation [166], competing for a common SRF docking site [127] and/or recruiting of corepressors such as HDAC histone deacetylase to the promoter region and prevents the chromatin activation for SMC transcription [138, 167], collectively these studies suggest that transcriptional repressors may play an essential role in the regulation of myocardin gene expression. Embryonic stem cells (ESC) are characterised by their ability to self renew indefinitely and maintain pluripotency, defined as potential to differentiate into cells of three germ layers. The ESC pluripotent state is maintained by network of transcription factors including Oct 4 (Octamer binding transcription factor 4), Sox2 (SRY (sex determining region) Box-2) and Nanog (Nanog Homeobox). Nanog is a major regulator of pluripotency in ESC. Nanog maintains the pluripotent state by suppressing the transcription of downstream genes for differentiation and activation of genes necessary for self renewal of ESC. LIF/Stat3 signaling rapidly upregulates KLF4 activating the direct downstream target KLF4 but not Oct4/Sox-2, which then forms a complex with Oct4 and Sox-2 and induces Nanog expression in embryonic stem cells. Withdrawal of LIF, resulted in drastic downregulation of KLF4 and both Oct4 and Sox2 was unable

to induce Nanog promoter, suggest that KLF4 binding on Nanog promoter region enhances the Oct4/Sox2 binding and activation of Nanog expression. KLF4 found to regulate Nanog expression by directly binding on its promoter region.

KLF4 is a key transcription factor for maintenance of self renewal and pluripotency in progenitor cells [345]. The shRNA mediated knockdown of KLF4 leads to differentiation of ESC [338] providing evidence of KLF4 –mediated control of pluripotent transcription factors and differentiation in progenitor cells. However, the mechanism of KLF4 mediated suppression of progenitor cells differentiation is unclear.

KLF4 is one of the most reported repressors associated with downregulation of SMC genes [138, 167]. KLF4 abrogates myocardin induced smooth muscle transactivation by physically interacting with SRF and inhibiting myocardin/SRF complex formation and attenuates smooth muscle gene expression [167]. However, to date there is no direct evidence for transcriptional regulation of myocardin gene expression by KLF4

Therefore, this study **hypothesis that KLF4 acts as a negative regulator for the myocardin promoter activity in hSMSPC cells**.

4.2. Methods and materials

4.2.1. PCR (Polymerase chain reaction) amplification and cloning of myocardial promoter region:

To amplify and clone a 3.9 Kb human myocardin promoter region from human genomic DNA, DNA was isolated by using the genomic DNA isolation kit (Promega, Wisconsin, USA) following the manufacture's instruction. Using Primer output 3 software (http://primer3.wi.mit.edu/), the PCR primers (forward 5'-*AAAGACCTGAATTCTATCAATAACC*-3' and the reverse 5'-*ACAACCCAGGATCCATCCAACTCTCCGTGA*-3') were designed to amplify 3.925 Kb of the human myocardin promoter. To clarify the region of PCR amplification, an *in silico* PCR using UCSC browser http://genome.ucsc.edu/cgi-bin/hgPcr) was carried out with the designed PCR primers, which amplified a 3.925 Kb (chr17:12565490+12569414) region on chromosome 17. A 3.925 Kb fragment of the human myocardin promoter was amplified by PCR using 50 ng of human genomic DNA and PCR mixture containing 1X high fidelity PCR buffer, 2.0 mM $MgSO_4$, 0.2 mM each dNTPs (Invitrogen, Carlsbad, CA), 0.4 µM human myocardin promoter primers (forward and reverse), 2.5 units of platinum taq DNA polymerase high fidelity (Invitrogen, Carlsbad, CA), 2.0 µl of DMSO (Sigma, St.Louis, MO) in 50 µl

reaction. The reaction was performed for 35 cycles of 30 seconds at 95°C, 30 seconds at 55°C, 4.5 minutes at 68°C in a thermocycler (Montreal Biotech Inc, Canada) PCR machine. The 3.9 kb PCR amplified fragment was eluted from 1% agarose gel by using the gel extraction kit (Qiagen, Hilden, Germany) and cloned into pGlow TOPO cloning vector (Invitrogen, Carlsbad, CA). The ligation reaction was carried out by following 1:3 vector/ insert ratio in 1X ligation buffer and 40U of ligase enzyme (New England Biolabs, Ipswich, MA) in 20 µl reaction volume at room temperature for 10 minutes and then transformed into One Shot TOP10 (Invitrogen, Carlsbad, CA) chemically competent cells by heat shock method. Positive clone was identified through colony PCR with the human myocardin forward and reverse primers. Restriction digestion of the positive clone with *EcoRI* and *BamHI* restriction enzymes confirmed the presence of PCR amplified 3.9 kb human myocardin promoter region and further sequencing confirmed the correct sequence and orientation of the human myocardin promoter region in a cloning vector.

4.2.2. Construction of reporter plasmids for screening human myocardin promoter activity *in vitro*

4.2.2.1. Construction of lentiviral plasmid vector containing human myocardin promoter driving eGFP expression.

The human myocardin promoter driving eGFP reporter lentiviral plasmid pHRSIN-ChMYOCDGW-dINotI referred in short as HR-ChMYOCDGW was based on pHRSIN-CSGW-dINotI referred as HR-CSGW vector which has spleen focus-forming virus (SFFV) promoter driving eGFP expression. The myocardin promoter replaces the SFFV promoter region in the HR-CSGW plasmid to generate HR-ChMYOCDGW. The HR-CSGW vector was cut with *EcoRI* /*BamHI* (New England Biolabs, Ipswich, MA) restriction enzymes to remove the SFFV promoter, then excised the vector backbone. For the insert, pGlow TOPO containing 3.925 Kb human myocardin promoter was cut with *EcoRI* /*BamHI* restriction enzymes, then the human myocardin promoter was excised as a 3.9 Kb *EcoRI–BamHI* fragment and ligated into the *EcoRI–BamHI* sites of HR-CSGW vector backbone to generate HR-ChMYOCDGW plasmid.

4.2.2.2. Construction of human myocardin promoter-luciferase reporter plasmid

The pGlow TOPO vector containing human myocardin promoter was cut with *BamHI,* to release the human myocardin promoter region (3.925 Kb), then

excised and ligated into *BglII* (*BamHI* cohesive termini compatible) linearised pGL3 Enhancer plasmid (Promega, Wisconsin, USA) to generate pGL3 E-hMyocdP plasmid. Eleven putative recombinant colonies were screened to detect the positive colony carrying human myocardin promoter (Fig 10.1). The correct sequence and orientation was confirmed using restriction enzymes and sequencing using the RV3 5' binding primer.The resulting sequence obtained from sequencing was named as seq1 and seq2 respectively. Further primers were designed within the sequenced regions seq1 and seq2 to sequence the remaining unsequenced human myocardin promoter within the pGL3 E vector. The sequences obtained from sequencing using primers within seq1 and seq2 was named as seq3 and seq4. The sequence for seq1, seq2, seq3 and seq4 were shown in (Fig 10.5). These sequences were aligned against the human genome using multialign software http://multalin.toulouse.inra.fr/multalin/cgi-bin/multalin.pl**).** The results showed one mismatch within the entire 3.925kb sequence. The mismatch was highlighted as (*) mark within the aligned sequence in (Fig 10.6).

4.2.3. Comparative sequence analysis of myocardin promoter

To identify the evolutionary conserved regions **(**ECRs) region conserved between species, ECR base program (http://ecrbrowser.dcode.org/) was used to compare mouse, rat and human myocardin promoter sequences. The putative transcriptional factors that bind to the 3.9 kb upstream region (-4007 to -100bp) of the human myocardin promoter was predicted using matInspector (www.genomatix.de) software with 0.75 cut-off core and matrix similarity.

4.2.4. Cell culture and transduction

4.2.5. Determination of optimal media and cell density for high transduction efficiency

To optimize media for hSMSPC transduction, hSMSPC were seeded at $5x10^4$ per cm^2 in 24 well plate in EGM2 media. The cells were allowed to settle for 16 hours and then transduced with 5 MOI of HR-CSGW lentivirus in different growth media such as EGM2, EGM2 0.5%FBS, EGM2 serum free (SF), SmGM2, SmGM2 SF, DMEM 10% FBS, DMEM SF and X-vivo media. After 24 hours, the growth media was removed and FACS analysis was performed as outlined in general methods section 2.8.

To optimize hSMSPC cell density for high transduction efficiency, hSMSPC cells were plated at range from 10,000 – 80,000 cells per cm^2 in 24 well plate in

EGM2 media. The cells were allowed to settle for 24 hours and then transduced with 5 MOI of HR-CSGW lentiviral particles. At day 1, the growth media was removed and FACS analysis was performed as outlined in general methods section 2.8.

4.2.5.1. Human myocardin promoter driven expression in cell types

To check the cell type specificity of activation of the human myocardin promoter driving eGFP (HR-hMYOCDGW), HASMC, hSMSPC and HUVEC cells were transduced with 5 MOI of HR-ChMYOCDGW or HR-CSGW lentivirus at a cell density of 50,000 cells per cm^2 in 24 well plate. At, day 4, growth media was removed and FACS analysis was performed as described in general methods section 2.8.

4.2.6. Optimisation of puromycin selection in hSMSPC (Puromycin kill curve analysis)

Puromycin drug resistance marker was used for generation of stable cells expressing the shRNA construct. Puromycin is a protein synthesis inhibitor used as a selective agent in cell culture to eliminate untransduced cells but it is toxic to cells. Therefore, kill curve analysis was performed to determine the minimal dose of puromycin needed to cause 100% cell death in untransduced hSMSPC cells. The cells were plated at $4x10^4$ cells per cm^2 cell density in EGM2 medium. The next day the growth media was changed with media supplemented with 0-15 µg/ml concentration range of puromycin. The cells were monitored daily and a phase contrast image was taken 24 hours after puromycin selection. The cells were washed with PBS, collected using 0.25% trypsin and suspended in EGM2 medium. The dead cells were stained with trypan blue and the percentages of viable cells were counted using a haemocytometer.

4.2.7. Puromycin at 1 µg/ml concentration causes massive cell death in non transduced hSMSPC cells

In order to evaluate the optimal dose of puromycin required to kill 100 % of untransduced hSMSPC cells, hSMSPC cells were incubated in range of puromycin (0-15 µg/ml) concentrations for two days. The result showed that hSMSPC cells were very sensitive for puromycin and started to lift within 24 hours of incubation (Fig. 4.1.A). The percentage of viable cells remained in puromycin containing media was counted after two days of puromycin incubation and plotted against corresponding puromycin concentration (Fig. 4.1.B). The result demonstrated that

puromycin at 1 µg/ml concentration caused 100% lethality in non transduced hSMSPC cells.

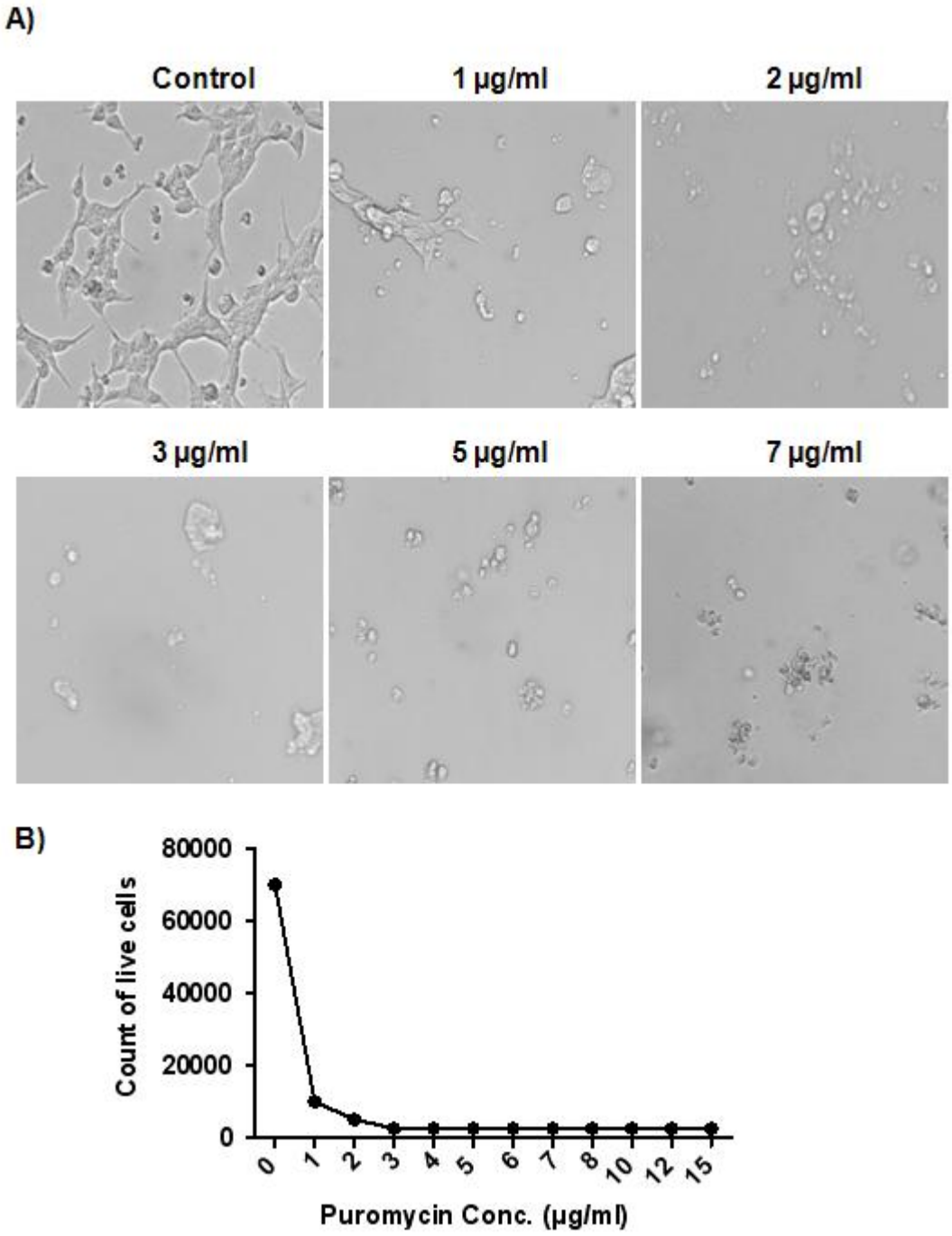


Figure 4-1. Optimisation of puromycin selection conditions for hSMSPC cells.

To determine the minimum dose of puromycin to cause 100% lethality in untransduced hSMSPCs, cells were plated at $7x10^4$ cells per well in 24 well plate in EGM2 media. The next day the cells were treated with various concentrations (0-10 µg/ml) of puromycin in EGM2 media. A) Puromycin at 1µg/ml caused massive cell death within 24 hours of treatment. B) Puromycin kill curve showing cell death in relation to increasing doses of puromycin over 48 hours in hSMSPC cells.

4.2.8. Construction of shRNA KLF4 lentiviral vectors

To screen potential LV_shKLF4 target sequences for efficient KLF4 knockdown, five different LV_shKLF4 sequences targeting different regions of human KLF4 mRNA as listed in (Fig.4.5A (i)), were used to generate shRNA expressing pKLO.1.puro-1 based lentiviral vectors. The two complementary oligos containing sense and anti sense strand connected by a spacer region capable of forming a short hairpin loop were synthesized using each LV_shKLF4 target sequence. In addition, the poly T termination signals sequence and *EcoRI* site was added to the shRNA sequence. The oligos were annealed to form dsDNA with a 5' sticky end (suitable for *AgeI* digested site) and a 3' end compatible for ligation with an *EcoRI* cut site, cloned into *AgeI/EcoRI* linearised pKLO.1-puro lentiviral vector. Lentivirus for each LV_shKLF4 plasmid was generated by following the protocol as outlined in general methods 2.9.

4.2.9. ShRNA-mediated KLF4 knockdown

To test the efficiency of designed LV_shKLF4 lentiviral vectors for KLF4 knockdown in hSMSPC cells, these cells were seeded at $5x10^4$ cells/cm^2 plating density a day before transduction in EGM2 medium. The next day, growth media was replaced and the cells were infected with each LV_shKLF4 lentiviral supernatant along with the empty vector with no shRNA (pKLO.1.puro-1) 24 hours after transduction puromycin (1µg/ml) was added to the cells. KLF4 expression was assessed three days post puromycin selection by qRT-PCR and western blot as described in general methods section 2.6 and 2.14.

4.2.10. TGF-β1 stimulation on shRNA-mediated KLF4 knockdown and human myocardin promoter activity in hSMSPC

To evaluate the LV_shKLF4 B mediated knockdown effect on human myocardin promoter activity; hSMSPC cells were seeded at 50,000 cells per cm^2 plating density 16 hours before transduction in EGM2 media and transduced with 5 MOI of HR-ChMYOCDGW lentiviral particles alone or cotransduced with LV_shKLF4 B lentiviral supernatant. In addition, hSMSPC cells were stimulated with or without differentiation media. At, day 4, growth media was removed and FACS analysis was performed as described in general methods section 2.8.

4.2.11. Transient Transfection and dual luciferase reporter assay

To measure human myocardin promoter activity over time, 24 hours prior to transfection, hSMSPC cells were seeded at $7x10^4$ cells per cm^2 plating density in EGM2 media. hSMSPC cells were cotransfected with pGL3 E–hMyocd promoter and renilla luciferase plasmid (outlined in general methods section 2.12) alone or coinfected with LV_shKLF4 B supernatant. The transfection media was changed 16 post transfection to differentiation media. Luciferase activity was measured at 24, 48 and 72 hours post TGF-β1 stimulation. The growth media was removed and replaced with 20 µl of 1X passive lysis buffer (PLB) made in sterile water (Promega, Wisconsin, USA) and kept in the orbital shaker (Microtitre plate Shaker SSM5; Stuart) for 15 minutes at 300 rpm. The cell lysate was transferred to 1.5 µl eppendorf tubes for measuring luciferase activity in luminometer (Promega, Wisconsin, USA). Dual luciferase reporter assay was performed as outlined in general methods section 2.13.

4.2.12. Primer designing and *in silico* PCR

To design unique PCR primers to amplify the predicted putative KLF4 binding sites in hSMSPC, the human myocardin promoter sequence flanking predicted KLF4 binding sites was used for designing ChIP PCR primers using primer 3 software. The PCR primers were subjected to *in silico* PCR (UCGC genome browser http://genome.ucsc.edu) against human genome and ensured uniqueness for each designed primer sets. The primer specificity was also checked by PCR amplification of hSMSPC genomic DNA for 30 cycles (94^OC for 45 sec, 62^OC for 30sec, and 72^OC for 20 sec). The PCR product was resolved on 1% agarose gel and visualised with ethidium bromide. No template DNA and water only were used as PCR negative controls.

4.2.13. Sonication and shearing analysis

To ensure optimal shearing of chromatin lengths between 200 to 1500 bp for ChIP assay in hSMSPC, hSMSPC cells were fixed with formaldehyde and homogenised using dounce homogenizer. Then, the cross linked DNA was sheared with the sonicator by following the optimal conditions (6 pulses, 50% duty cycle and 30% output) on ice. A portion of lysate was used to check the DNA shearing efficiency. The lysate was heated at 65^OC overnight to reverse the cross links and subjected to RNase treatments for 15 min at 37^OC followed by proteinase K for 15 min at 67^OC, and DNA was recovered by using QIA quick purification kit (Qiagen).

The efficiency of chromatin shearing was assessed by loading 1 µg and 2 µg of sheared hSMSPC DNA on agarose gel and visualised with ethidium bromide.

4.2.14. ChIP assay

The chromatin immunoprecipitation (ChIP) assay was carried out in hSMSPC cells with antibodies against KLF4 (SC- 20691, Santa Cruz) or normal IgG (rabbit IgG, 3703, Prosci incorporated) following protocol mentioned in general methods section 2.15.

4.2.15. Real time PCR

To check whether co immunoprecipitation of KLF4 bind to myocardin promoter region, ChIP-PCR was performed using lightcycler 480 with SYGR green I with the primer pairs flanking putative binding sites S1 to S5 as listed in Fig 5.1.B. The input DNA was diluted to 1:10 ratio prior to PCR amplification and the reaction (20µl) mixture contained 2X PCR light cycler 480 SYGR Green I mastermix, 10µM of each forward and reverse primer, chromatin immunoprecipitated DNA as template was subjected to 40 cycles (94^{O}C for 20 sec, 59^{O}C for 30 sec, 72^{O}C for 30 sec) of PCR amplification. The fold enrichment was normalised to IgG (no antibody) control signal.

4.3. Results

4.3.1. Plating density at $5x10^4$ cells per cm^2 enhances hSMSPC transduction efficiency

To evaluate the optimal cell density for hSMSPC transduction, varying cell densities ranging from $1x10^4$ to $8x10^4$ cells per cm^2 were plated in EGM2 medium. hSMSPC cells were transduced with 5 MOI of HR-CSGW lentivirus vector and 24 hours of post transduction, FACS analysis for eGFP was carried out to determine optimal cell density for hSMSPC transduction. The results showed that transduction efficiency of hSMSPC increased with increasing cell density and reached a maximum transduction efficiency of approximately 39% at $5x10^4$ cells per cm^2 (Fig.4.2A). However, further increases in cell density showed a decline in hSMSPC transduction potential; suggesting that, hSMSPC cell density at $5x10^4$ cells per cm^2 was the optimal plating density for efficient hSMSPC transduction.

4.3.2. Serum containing EGM2 medium enhances hSMSPC transduction efficiency

In order to optimize the best growth media condition that facilitates high hSMSPC transduction efficiencies, different growth media such EGM2, EGM2 0.5% FBS, EGM2 SF, SmGM2, SmGM2 SF, DMEM 10% FBS, DMEM SF or X-VIVO media for hSMSPC cell transduction was compared. The hSMSPC cells were transduced at 5MOI of HR-ChMYOCDGW in the different growth media and it was found that basal EGM2 medium (with 2% serum) showed the higher transduction rates (42% Fig.4.2B) at 24 hours post transduction compared to serum free media with 31% of transduction. In addition, EGM2 0.5% FBS showed 37% transduction efficiency as compared to serum free EGM2 suggesting that small variations in FBS levels affect the hSMSPC transduction potential. The transduction efficiency of SmGM2 (27%), SmGM2 SF (11%), DMEM 10% FBS (18.6%), DMEM SF (20%) or X-VIVO (20%) conditions yielded much lower transduction rates (Fig.4.2B). These findings indicate that serum containing EGM2 medium enhances efficient lentivirus transduction into hSMSPC cells.

4.3.3. Human myocardin promoter driven eGFP reporter gene expression is restricted to HASMC and hSMSPC

The present study aims to determine the selective transcriptional activation of human myocardin promoter driving eGFP reporter expression in different cell types. The schematic representations of self replicating vectors were shown in Fig. 4.3A. To evaluate the ability of selective activation of the human myocardin promoter in HASMC, hSMSPC and HUVEC, cells were infected with to 5 MOI of HR-ChMYOCDGW or HR-CSGW for 4 days. The HR-CSGW was used to assess the transduction efficiency and permissivity of lentivirus vector in HASMC, hSMSPC and HUVEC cells. Expression of eGFP was detected by confocal microscopy and further verified using flow cytometry. Flow cytometry analyses of eGFP expression showed ubiquitous expression of eGFP in HR-CSGW transduced hSMSPC, HUVEC and HASMC cells (Fig.4.3B) with 69.8%, 52.8% and 46.6% of transduction efficiencies (Fig.4.3D) respectively; suggesting that hSMSPC, HASMC and HUVEC have no restriction for entry or interaction of lentivirus expression. However, human myocardin promoter from HR-ChMYOCDGW transduced lentivirus selectively activated eGFP expression in HASMC and hSMSPC but not in HUVEC (Fig.4.3C&D). These results suggest that human myocardin promoter directed eGFP expression was specific to smooth muscle cells, and not endothelial cells.

4.3.4. Identification of three Evolutionary Conserved Regions (ECRs) within the myocardin promoter of human, mouse and rat

The goal of this study was to identify any ECRs from alignments of the mouse and rat promoter sequences with the 3.9 kb human myocardin promoter sequence using web based ECR browser outline in 4.2.3 of methods. Using ECR browser parameters of at least 70% homology over 100 bps and the human genome as the base genome, three highly conserved ECRs within the human myocardin promoter (highlighted in brown box Fig. 4.4.A) were identified The sequence alignment was colour coded to indicate intergenic (red), UTRs (yellow) and transposons and simple repeats (green). The distal ECR sequence with transposons and simple repeats was referred as R1, the middle intergenic region was labelled as R2 and the ECR sequence near to the translational start site (+1) comprising of UTRs and intergenic sequence was labelled as R3 (Fig.4.4A.)**.** Examination of ECRs conserved between human and rat showed the alignment of rat sequence to -461/-101, -1526/-1409 and -2089/-1821 position on human myocardin promoter. The summary of the three ECR regions of the human myocardin promoter with rat and mouse was depicted in table 4.1. The ECR sequence was submitted to rVista 2.0 to identify potential conserved transcription factor binding sites (TFBS) and compared against matrices of TRANSFAC professional V10.2 library optimized for 0.85 matrix similarity. The results showed four TFBS conserved in R1 sequence and 62 TFBS in R3 sequence that are conserved between human and rat whereas the R2 sequence showed no conserved TFBS in the analysis**.** The dynamic overlay of potential TFBS for R1 and R3 is shown in Fig.4.4. (B & C).

Table 4-1. ECR conserved region between rat, mouse and 3.9 kb upstream of human myocardin promoter.

ECRs [100 bps] from the chr17:12565499-12569405 region
Rat [rn4]

	Relative position	Type	Genomic position	Length	Percent identity
1	1919-2127		chr17:12567417-12567685	269	70.6%
2	2482-2598		chr17:12567980-12568096	117	71.8%
3	3547-3907		chr17:12569045-12569405	361	84.8%
Mouse [mm10]					
	Relative position	**Type**	**Genomic position**	**Length**	**Percent identity**
1	1911-2181		chr17:12567409-12567679	271	70.5%
2	2443-2585		chr17:12567941-12568083	143	67.8%
3	3530-3907		chr17:12569028-12569405	378	83.3%

4.3.5. Prediction of putative transcription factors involved in the transcriptional regulation of human myocardin promoter

The goal of the promoter analysis was to identify the key regulatory elements involved in the transcriptional regulation of myocardin gene expression. Using genomatrix software [346] the human myocardin promoter sequence (cloned 3.925 Kb) was screened for putative transcription factor elements. The complete list of predicted transcription factors is given in an appendix**.** This analysis revealed putative bindings sites for cardiac transcription factors (GATA1, GATA3, NFAT, Nkx2.5, TBX), skeletal muscle transcription factors (MyoD, Myf5, Myogenin) and smooth muscle regulatory elements (SRF, GKLF, Elk1, HES1, twist, AML3 (Runx2), RBPJκ, YY1). Notably, the myocardin promoter has stem cell pluripotency associated transcription factors Nanog, OCT4, Sox2 and cMyc putative binding sites within its promoter with high core similarity scores. In addition, the results showed Isl1, NFkappaB and p53 putative binding sites within the human myocardin promoter.

While exploring the putative transcription factors for possible transcriptional repressors of myocardin gene expression, putative binding sites for GKLF, Elk1, HES1, twist, AML3 (Runx2) and YY1, (listed in table 4.2) were found, suggesting a possible mechanism for myocardin repression in hSMSPCs. Out of these repressors, KLF4 is a well known transcriptional repressor reported to suppress myocardin induced smooth muscle gene activation in SMCs [167]. The qRT-PCR results showed that hSMSPCs express low levels of myocardin and higher levels of KLF4. Thus, it is possible that the excess KLF4 may act as a transcriptional repressor regulating myocardin gene expression in hSMSPCs.

Therefore, the present study investigated the possibility of KLF4 repressing myocardin gene expression in hSMSPC cells. KLF4 has five different putative binding sites within the human myocardin promoter with high core similarity scores. The graphical representation of the five putative binding sites within the human myocardin promoter is shown in Fig. 4.5.A. The predicted KLF4 binding site 1 (-569) on (-) stand, site 3 (-3702) on (+) stand and site 5 (-3792) have a high core similarity 1 and 0.975, 0.88 and 0.966 matrix similarity respectively. The KLF4 putative binding site 2 located at (-1093) on (-) strand and site 4 (-3774) on (+) strand have 0.83 core similarity and 0.87 matrix similarity respectively (Fig. 4.5.B). The exact location of predicted KLF4 binding sites sequences within the human myocardin promoter sequence is shown in Fig. 4.5.B.

4.3.6. LV_shKLF4 B target sequence efficiently silence KLF4 expression in hSMSPC

To evaluate the functional relationship between the human myocardin promoter and transcription factor, KLF4, five different short hairpin lentiviral constructs were generated targeting different 18-23bp sequences of KLF4 mRNA as shown in Fig. 4.6. A (i&ii). The schematic representation of the physical location of five LV_shKLF4 sequences in human KLF4 mRNA was shown in Fig. 4.6. A(i). The actual shRNA target site and target sequences were depicted in Fig. 4.6. A(ii). The schematic diagram of the lentiviral vector (pKLO.puro.1) containing short hairpin RNA for KLF4 is shown in Fig. 4.6B. Out of the five sequences, LV_shKLF4 B construct showed 65% and < 90% silencing of KLF4 as analysed by qRT-PCR and western blotting respectively, in hSMSPC cells (Fig. 4.7 A-C) and therefore, it was selected for subsequent KLF4 knockdown experiments.

Table 4-2. List of putative binding sites for smooth muscle repressor transcription factors on human myocardin promoter

Matrix Family	Matrix	Position	Core Sim.	Sequence CAPITALS: Core Sequence
V$HAML	V$AML3.01	-2707/-2721	1	gagtGTGGtgtgtgt
V$HAML	V$AML3.01	-1309/-1323	1	cagaGTGGtttttat
V$HAML	V$AML3.01	-585/-599	1	gcagGTGGttcgcgg
V$YY1F	V$YY1.02	-3712/-3732	1	cccaaCCATctccaaacattc
V$YY1F	V$YY1.01	-3262/-3282	1	taactCCATtttacagataag
V$YY1F	V$YY1.02	-1966-1986	1	ttcctCCATttttgaagccag
V$YY1F	V$YY1.01	-337-357	1	ctttgCCATctgatacccttt
V$KLFS	V$GKLF.02	-3776/-3792	1	cagcAAAGgagagaaag
V$KLFS	V$GKLF.01	-3758/-3774	0.826	caaaatgaaGGGGaaag
V$KLFS	V$GKLF.01	-3686/-3702	1	gtaaaaagaAGGGtcca
V$KLFS	V$GKLF.01	-1077/-1093	0.826	aaatagggaGGGGctct
V$KLFS	V$GKLF.01	-553/-569	1	aaagaaggaAGGGctgc
V$ETSF	V$ELK1.01	-259/-279	1	atcagacaGGAAcgcctggga
V$HESF	V$HES1.01	-734/-748	0.944	agggcgtGCGCgccc
V$HESF	V$HES1.01	-638/-652	0.944	tgggcgtGCGCagcc
V$HAND	V$TWIST.01	-3323/-3343	0.952	aagaataCATGtggggtgccc

4.3.7. Regulation of myocardin gene expression by KLF4 protein.

Previous studies showed that the myocardin promoter can drive eGFP expression in hSMSPC and in HASMC, but not in HUVEC cells (Fig. 4.3), and moreover results from cytokine stimulation studies reported that TGFβ1 stimulates SMC differentiation of hSMSPCs (Fig. 3.5.). To test whether KLF4 directly regulates myocardin gene expression, hSMSPC cells were infected with HR-ChMYOCDGW alone or cotransduced with LV_shKLF4 B supernatant and stimulated with or without TGF-β1 (5ng/ml) in 1% SmGM2 media for 4 days. Expression of eGFP was detected on day 4 using confocal microscopy Fig. 4.8A. Flow cytometry analysis showed no obvious increase in human myocardin promoter directed eGFP expression with TGF-β1 stimulation as compared to basal myocardin promoter activity. However, KLF4 knockdown resulted in a two fold increase in myocardin promoter directed eGFP expression (Fig 4.8 i&ii), indicating that myocardin transcriptional machinery exists and is repressed by the presence of KLF4 expression in hSMSPC. Notably, TGF-β1 stimulation resulted in increased induction of human myocardin promoter activity when compared to human myocardin promoter activity in KLF4 knockdown hSMSPC cells. However, this increase was not statistically significant, suggesting that myocardin promoter-driven gene expression is repressed by KLF4 in hSMSPC cells.

4.3.8. KLF4 silencing and time course of human myocardin promoter activation in hSMSPC

Previous results showed that knockdown of KLF4 in hSMSPC significantly increased myocardin promoter directed eGFP expression by day 4 post transduction. To further investigate the pattern of myocardin promoter activation upon TGF-β1 stimulation over time, a time course analysis using human myocardin promoter luciferase reporter construct was performed. hSMSPC cells were first transduced +/- LV_shKLF4 B lentiviral construct. The next day the cells were co transfected with human myocardin promoter luciferase reporter construct and pRL-TK renilla luciferase construct, then stimulated with TGF-β1 (+/-) for 24, 48 and 72 h respectively.

The human myocardin promoter directed luciferase activity was slightly increased at 48 h post transfection, but then significantly decreased below basal levels at 72 h post transfection in hSMSPC cells. However, human myocardin promoter activity showed a drastic increase in promoter activity (nearly doubled) at

48 h post transfection and reached baseline promoter activity at 72 h post transfection in KLF4 knockdown hSMSPC cells (Fig 4.9. B-D).

TGF-β1 stimulation, slightly increased human myocardin promoter activity as compared to baseline promoter activity 24 h post stimulation but then a sustained repression was observed 48, 72 h post stimulation. Although, human myocardin promoter activity was decreased 48 h post stimulation, a significant increase in promoter activity was observed 72 h post stimulation in KLF4 knockdown hSMSPC cells. However, there was no significant difference in human myocardin promoter activity observed between unstimulated and TGF-β1 stimulated hSMSPC as well as KLF4 knockdown hSMSPC cells. These results again support KLF4 as a transcriptional repressor for myocardin gene expression in hSMSPC cells (Fig 4.9. B-D).

4.3.9. QRT-PCR primer designing for chip-PCR assay

To analyse KLF4-bound DNA sequence in immunoprecipitated samples by qRT-PCR, primers were designed to amplify approximately 200 bp of human myocardin promoter that contained putative KLF4 binding sites. The schematic representation of putative KLF4 binding sites (grey elongated circles) within the human myocardin promoter along with the direction and location of designed PCR primer (shown by arrows) and its amplicon size (brown bars) were shown in Fig. 4.11.A. As a first step, the designed PCR primers were subjected to *in silico* PCR (UCSC browser) against human genome and found that the designed primers for ChIP assay were unique to myocardin promoter sequence. Further, the specificity of designed ChIP-PCR primers was also tested by PCR amplification with primers listed in Fig.4.11.B on genomic DNA isolated from hSMSPC cells. The hSMSPC genomic DNA was amplified as single amplicon for each primer pairs of expected sizes as shown in (Fig.4.12.A); suggest that the designed PCR primer is unique to each putative KLF4 binding site on human myocardin promoter.

4.3.10. Sonication of hSMSPC genomic DNA for six pulses yielded optimal sheared chromatin for ChIP assay

To optimize chromatin shearing for ChIP assay using sonicator, hSMSPC ($8x\ 10^6$) cells were scrapped in ice cold PBS and fixed with 1% formaldehyde in EGM2 media. The cross-linked chromatin was homogenized and the pellet was collected after centrifugation. The pellet was suspended in shearing buffer and subjected to six pulses of sonication using sonicator. Each cycle consisted of 30 sec of sonication with 50% duty cycle and 30% output followed by one minute incubation

on ice. The sheared DNA was subjected to reversal of cross links and subsequent proteinase K and RNase A treatments. Then, DNA was recovered using QIA quick spin column kit. The efficiency of sonicated DNA was tested by electrophoresis through a 1% TAE agarose gel. The resolved sheared chromatin Fig. 4.12.B showed a smear of DNA fragments ranging between 200 to 1500 bp optimal for ChIP assay indicates that hSMSPC chromatin sonicated for six pulses generated chromatin fragments optimal for ChIP assay.

4.3.11. KLF4 binds to human myocardin promoter in cultured hSMSPC cells

To further test how KLF4 regulates human myocardin gene expression in hSMSPC cells, ChIP assay was performed to confirm the KLF4 binding within the human myocardin promoter in hSMSPC cells. For ChIP assay, hSMSPC cells were fixed with formaldehyde to cross link the protein-DNA complexes within the cells. Then, DNA was sheared to smaller fragments ranging between 200 to 1500bp by sonication (Fig.4.12B.). An aliquot of sheared DNA was taken before immunoprecipitation and used as reference sample (INPUT sample). The sheared chromatin was immunoprecipitated with antibody against KLF4 or normal IgG. The immunoprecipitated DNA along with the INPUT sample was then reversed cross links and purified using proteinase K treatment. The purified immunoprecipitated DNA and INPUT samples were analysed by 40 cycles of PCR amplification using SYBR green I with specific primers pairs corresponding to five putative KLF4 binding regions of the human myocardin promoter as listed in Fig.4.11B. It is clear that the region corresponding to the putative KLF4 binding site 3 (-3702) was significantly enriched in the immunoprecipitated chromatin as compared with other predicted KLF4 binding sites (site-1, 2, 4 & 5) on myocardin promoter from the hSMSPC cells (Fig. 4.13). In support of this, primer set designed to amplify the clustered potential KLF4 binding site (site 3, 4 & 5) also showed significant levels of enrichment as measured by RT-PCR analysis. Taken together, these studies indicate KLF4 can directly binds to the human myocardin promoter at position -3702 to -3686 on (+) stand upstream of translation start site (TSS) and inhibits human myocardin gene expression in hSMSPC cells. Apart from KLF4, we also found putative binding sites for other transcription factor involved in the regulation of myocardin gene expression within the human myocardin promoter (Fig 4.10).

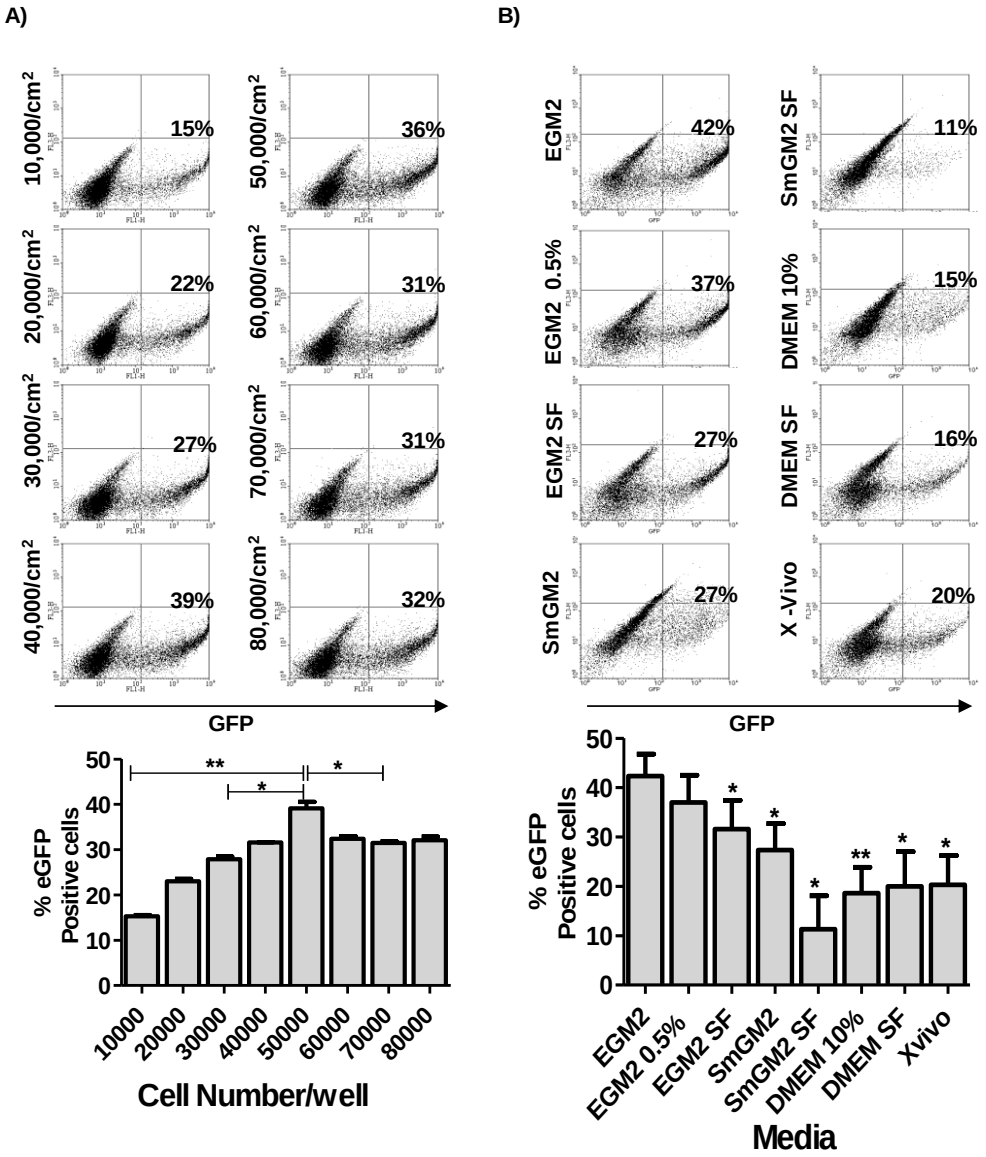
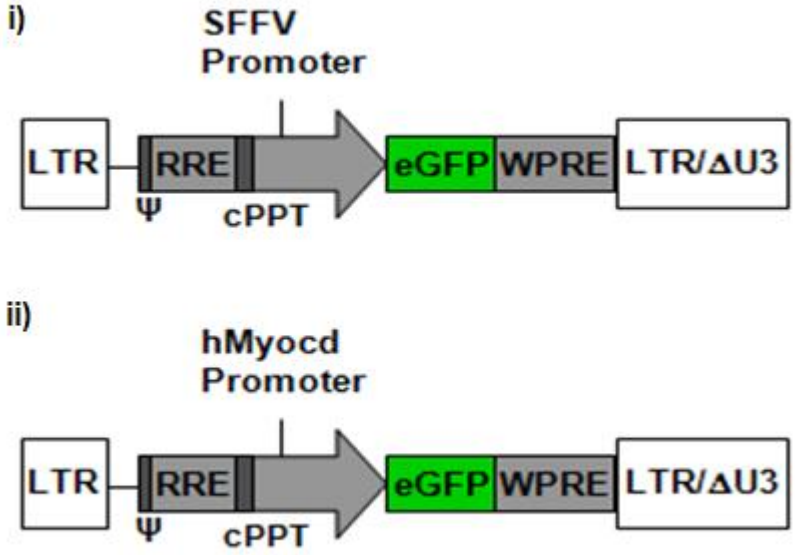


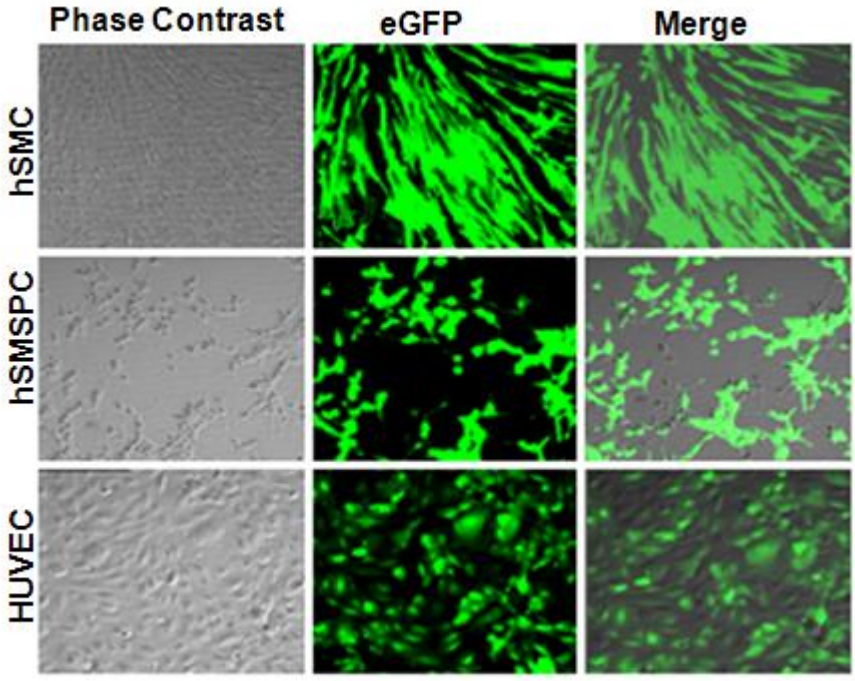
Figure 4-2. Effect of cell density and growth media on the transduction efficiency in hSMSPC cells.

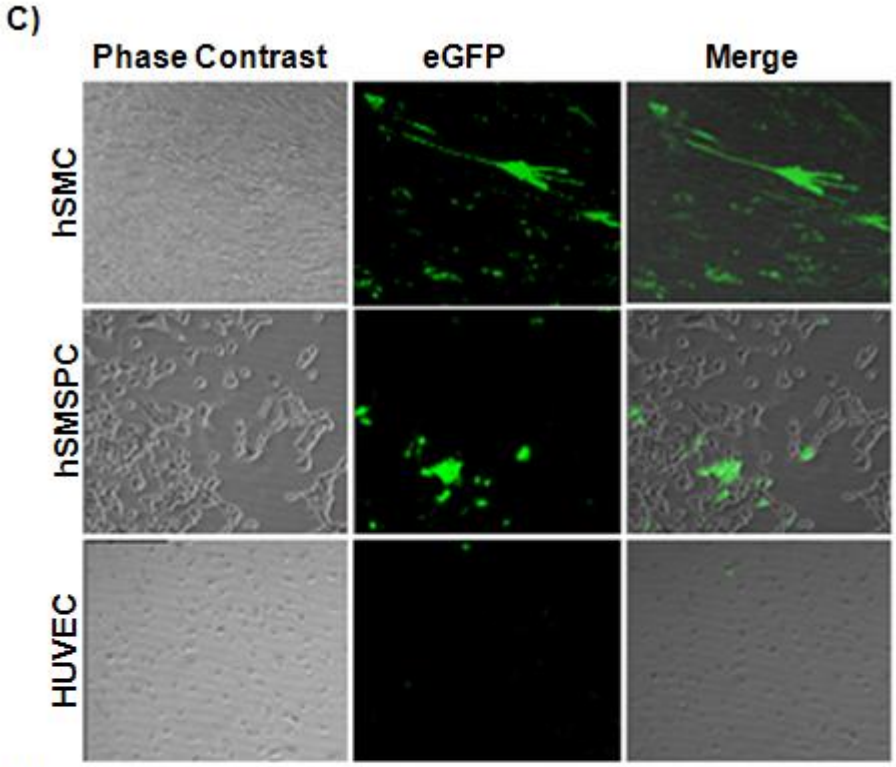
A) To evaluate the effect of cell density on hSMSPC transduction, cells were grown at different cell densities in EGM2. The next day the cells were transduced with 5MOI of lentivirus expressing eGFP (HR-CSGW) vector. After 24 hours FACS analysis shows high percentage of eGFP expression in cells plated at $5x10^4$ cells per cm^2 cell density. B) To evaluate whether growth media has effects on hSMSPC transduction potential, cells were plated at $5x10^4$ cells per cm^2 in 24 well plate in EGM2 media. The next day transduction was done with 5MOI of HR-CSGW vector in different growth media including EGM2, EGM2 0.5% FBS, EGM2 SF, SmGM2, SmGM2 SF, DMEM 10%, DMEM SF or X-Vivo medium. After 24 hours of transduction, FACS analysis was performed for eGFP expression. EGM2 media shows high percentage of eGFP positive cells. Data are representative of three independent experiments. n=3, SF- Serum Free. ** P<0.01, *P<0.05.

A)


i)
SFFV
Promoter
LTR
RRE
Ψ
cPPT
eGFP
WPRE
LTR/ΔU3
ii)
hMyocd
Promoter
LTR
RRE
Ψ
cPPT
eGFP
WPRE
LTR/ΔU3

B)


Phase Contrast
eGFP
Merge
hSMC
hSMSPC
HUVEC


C)
Phase Contrast
eGFP
Merge
hSMC
hSMSPC
HUVEC

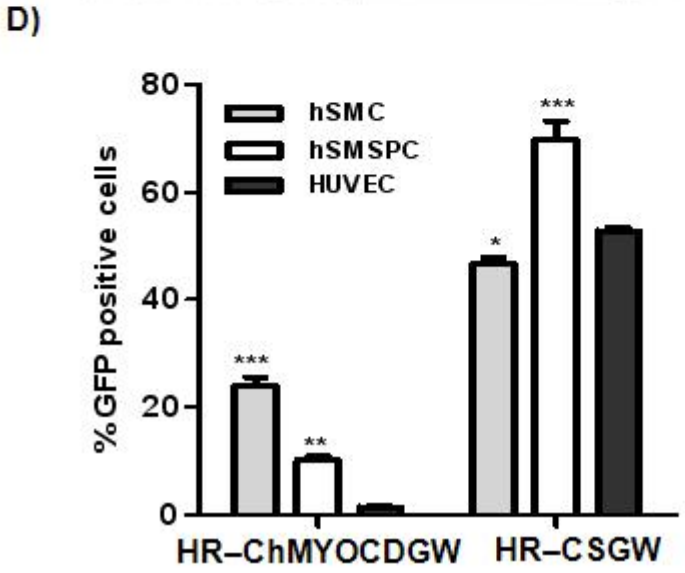
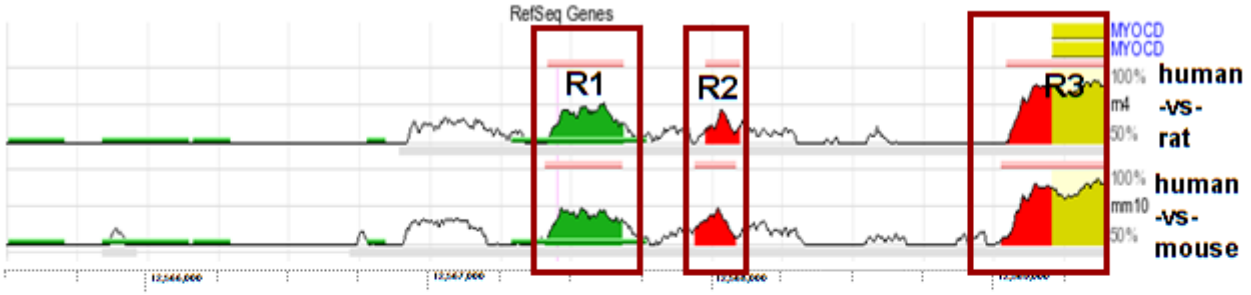

D)
80
60
40
20
0
%GFP positive cells
hSMC
hSMSPC
HUVEC
***
**
*
***
HR–ChMYOCDGW
HR–CSGW

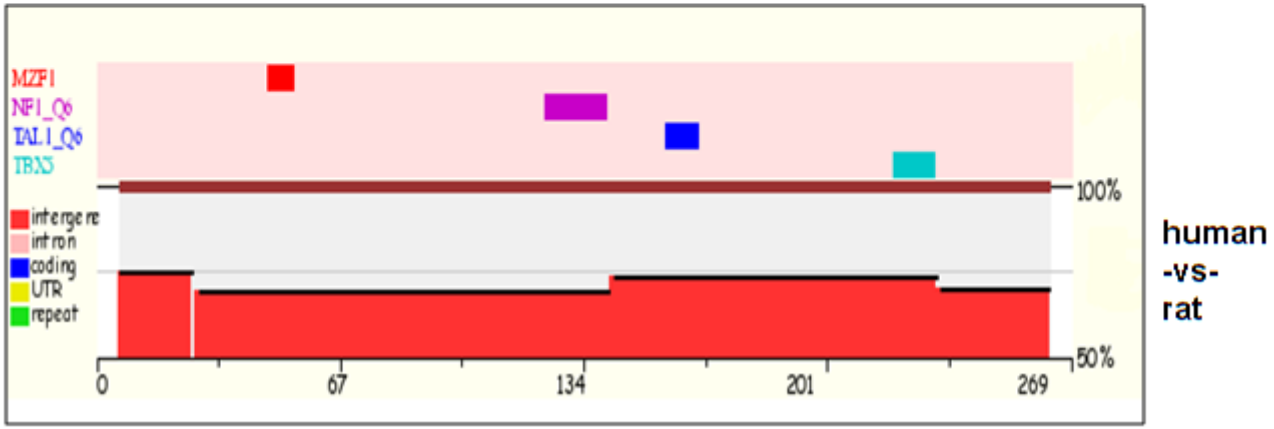
Figure 4-3. Construction and characterisation of human myocardin promoter construct.

A) Schematic representation of self replicating (SIN) second generation lentiviral vector. (i) pHRSIN-CSGW-dINotI (HR-CSGW) vector with SFFV promoter (ii) pHRSIN-ChMYOCDGW-dINotI (HR–ChMYOCDGW) vector with human myocardin promoter. LTR, Long terminal repeat; SFFV, spleen focus-forming virus (promoter); Ψ, packaging signal; RRE, Rev response element; cPPT, central polypurine tract; WPRE, Woodchuck hepatitis virus post-transcription regulatory element. To evaluate the selective activation human myocardin promoter directed eGFP expression in different cell types, HASMC, hSMSPC and HUVECs were plated at $5x10^4$ cells/cm^2 seeding density in EGM2 media. At 24 h post seeding, cell types were transduced with 5 MOI lentiviral vector encoding SFFV (HR-CSGW) promoter expressing eGFP (act as a positive control for vector integration) and 5MOI lentiviral vector encoding human myocardin promoter expressing eGFP (HR-ChMYOCDGW) vector. B) Ubiquitous expression of HR–CSGW vector in all three, HASMC, hSMSPC and HUVEC cell types. C) Selective expression of HR–ChMYOCDGW vector only in HASMC and hSMSPC but not in HUVEC. D) Day 4 FACS analysis of cell types transduced with lentiviral vector encoding SFFV and hMyocardin promoter with eGFP expression. Values are the means ± SD of three independent experiments. n=3, *** P<0.001, **P<0.01, *P<0.05

A)


RefSeq Genes
MYOCD
MYOCD
R1
R2
R3
100%
human
-vs-
rat
50%
100%
human
-vs-
mouse
mm10
50%

B)


MZF1
NF1_Q6
TAL1_Q6
TBXT
intergene
intron
coding
UTR
repeat
100%
50%
0
67
134
201
269
human
-vs-
rat

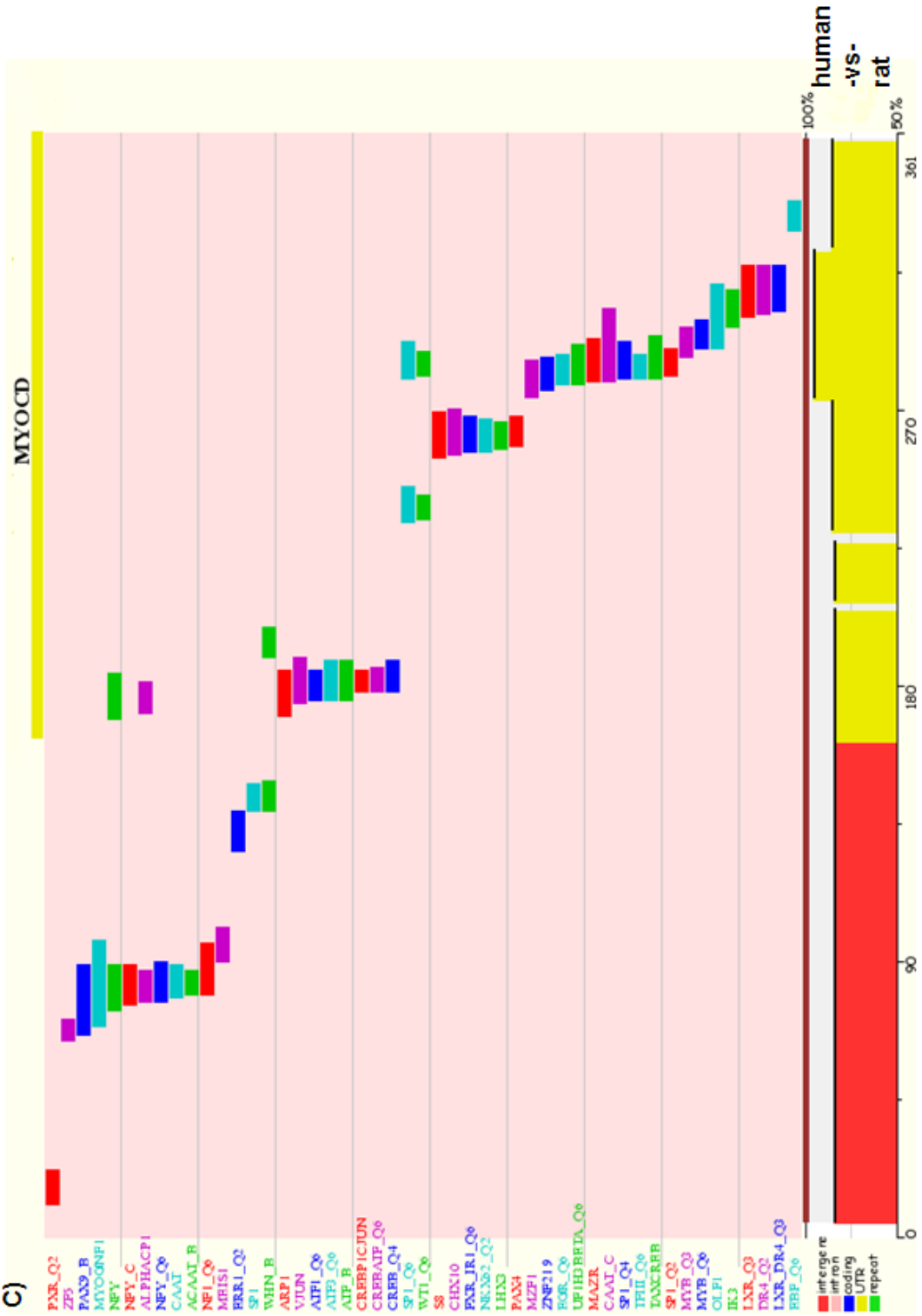


Figure 4-4. ECRs in the myocardin promoter.

A) Comparative genome analysis of myocardin promoter sequences between human rat, and mouse revealed three highly conserved ECR which was highlighted in brown box and labelled as R1, R2 and R3 respectively. Red peak corresponds to intergenic conserved region, green peak corresponds to transposable elements and simple repeats and yellow corresponds to UTRs. B) Prediction of conserved transcription factors within region R1 between human and rat. C) Prediction of conserved transcription factors within region R3 between human and rat**.**

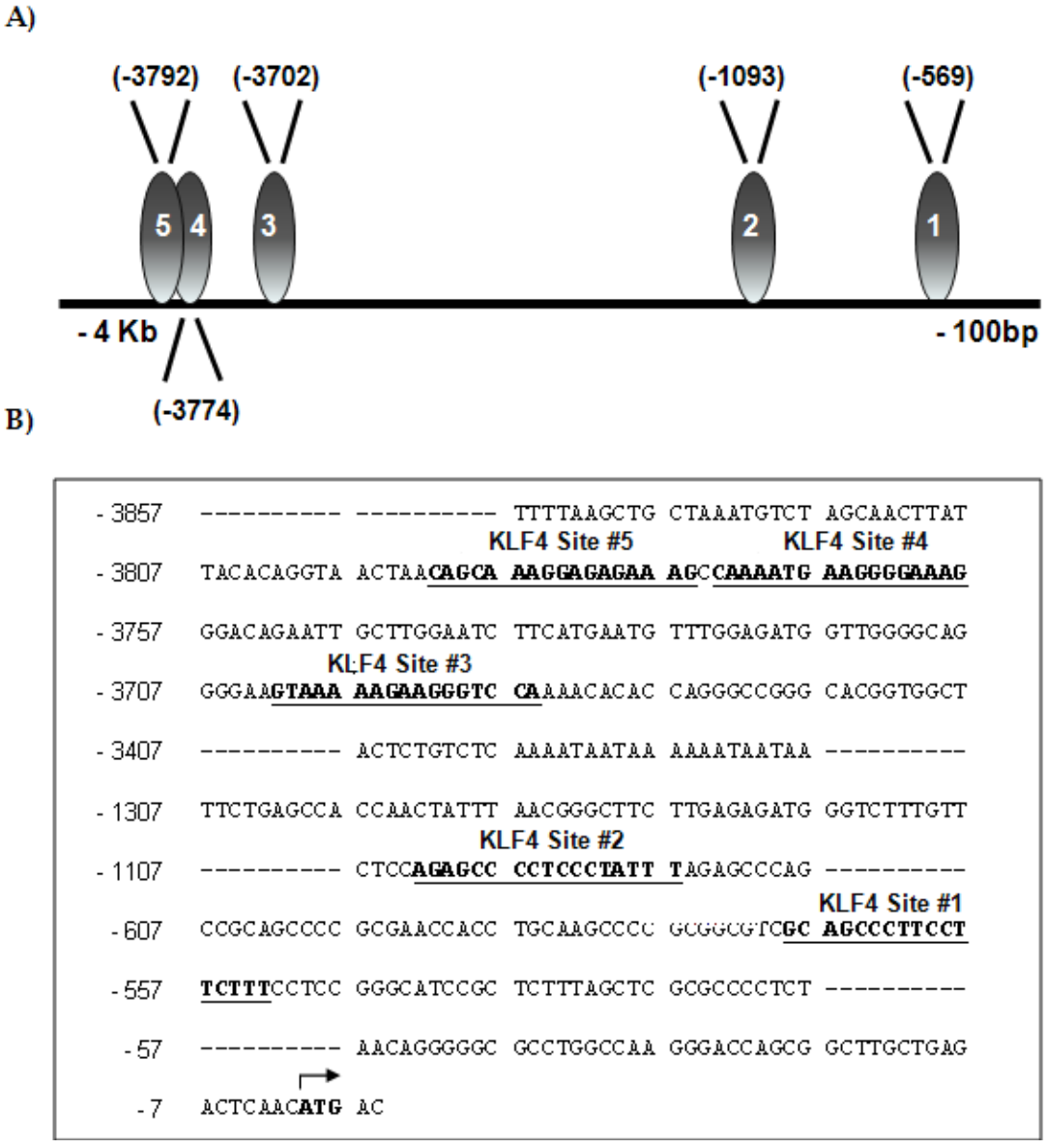
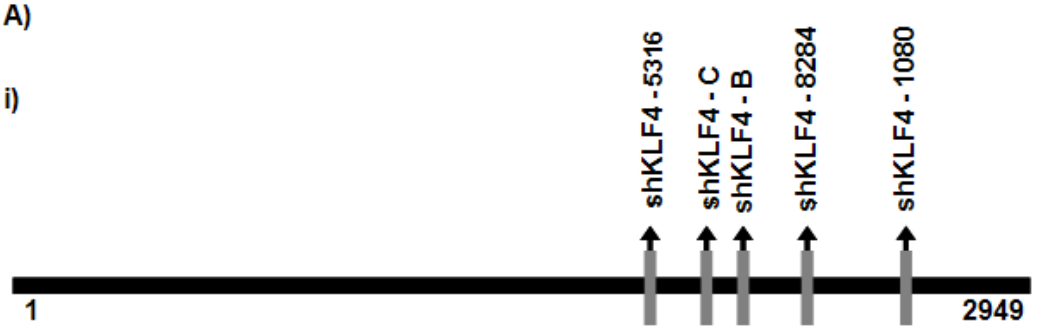


Figure 4-5. The human myocardin promoter has five putative KLF4 binding sites that potentially regulating human myocardin gene expression.

A) The schematic representation of human myocardin promoter (-100bp to -4kb relative to translational start site) containing five putative KLF4 binding sites (the position of putative KLF4 binding sites are represented by semicircles). The consensus KLF4 binding sequences are also shown. B) A schematic representation of upstream nucleotide sequence of human myocardin promoter with the position of putative binding sites for KLF4. The putative binding sites are underlined and labelled as site KLF4 #1 – 5 relative to TSS. The translation start site is indicated by black bent arrow.

A)

i)



ii)

S.No	shRNA	Target sites on KLF4 mRNA	Target sequence
1	shKLF4 5316	1680-1700	GCTCCATTACCAAGAGCTCAT
2	shKLF4 C	1821-1843	GAGTTCCCATCTCAAGGCACACC
3	shKLF4 B	1884-1902	GGACGGCTGTGGATGGAAA
4	shKLF4 8284	2032-2052	TAAATCCCAGACAGTGGATAT
5	shKLF4 1080	2320-2340	TTGTGGATATCAGGGTATAAA

B)

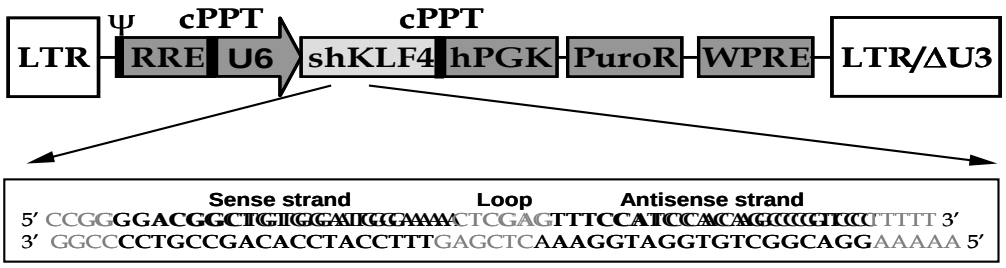


Figure 4-6. Screening and construction of a shRNA lentiviral vector targeting human KLF4 expression.

A) Selection of target sites within the KLF4 gene. i) Graphical representation of physical position of five selected target shKLF4s on KLF4 mRNA sequence. The shKLF4s 5316, B, C and 8284 were localised in same location on coding region expect shKLF4 1080 located in 3'UTR region. ii) The table shows the exact location of five selected shKLF4 target sites and their sequence on mRNA of KLF4. B) A Schematic diagram of the lentiviral vector (pKLO.puro-1) containing short hairpin RNA for KLF4. The shKLF4 was driven by U6 promoter and the vector encodes puromycin resistance gene ($Puro^R$) driven by human phosphoglycerate kinase eukaryotic (hPGK) promoter. The vector harbours LTR, Long terminal repeat,Ψ - packaging signal; RRE, Rev Response element; cPPT, central polypurine tract. Schematic depiction of the complementary forward and reverse oligos designed for shKLF4 B sequence with poly T termination sequence and *EcoRI* site as shown in the below panel. The sense and antisense shKLF4 B sequences were shown as bold characters has spacer capable of forming loop and cloned into *AgeI/EcoRI* site of the vector.

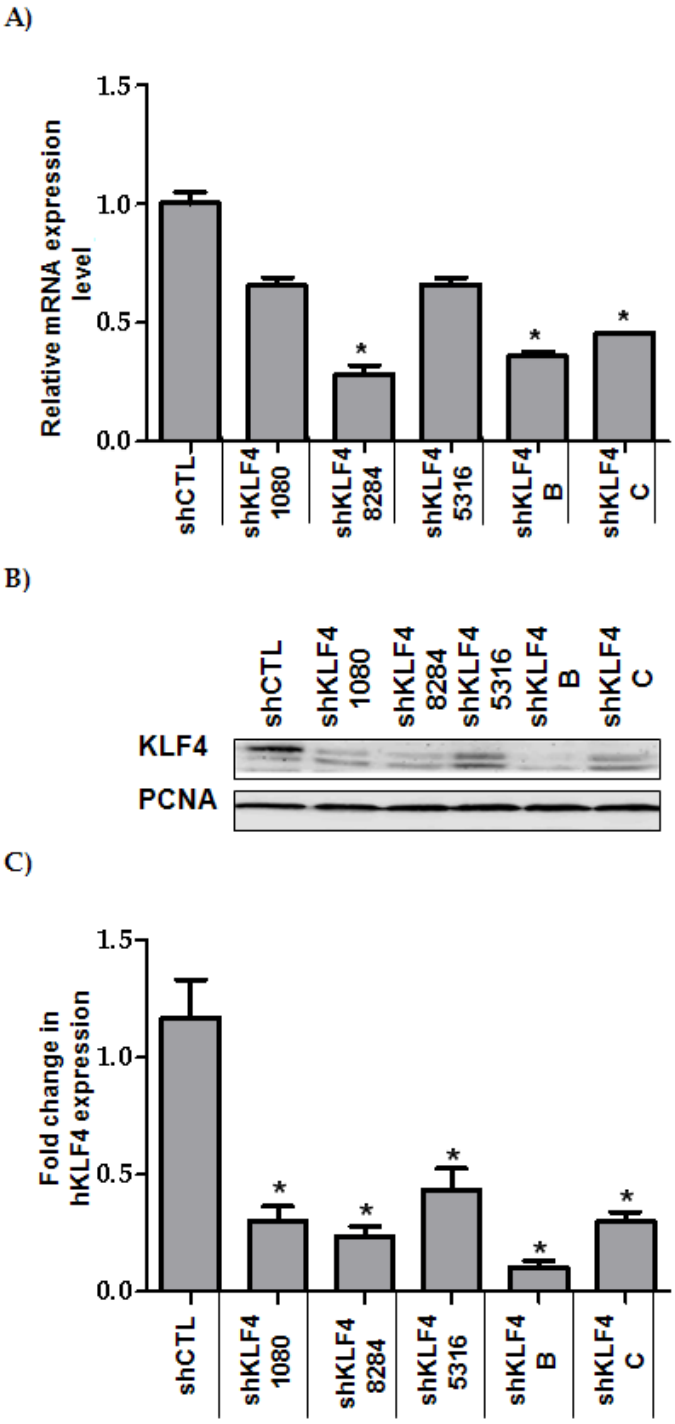
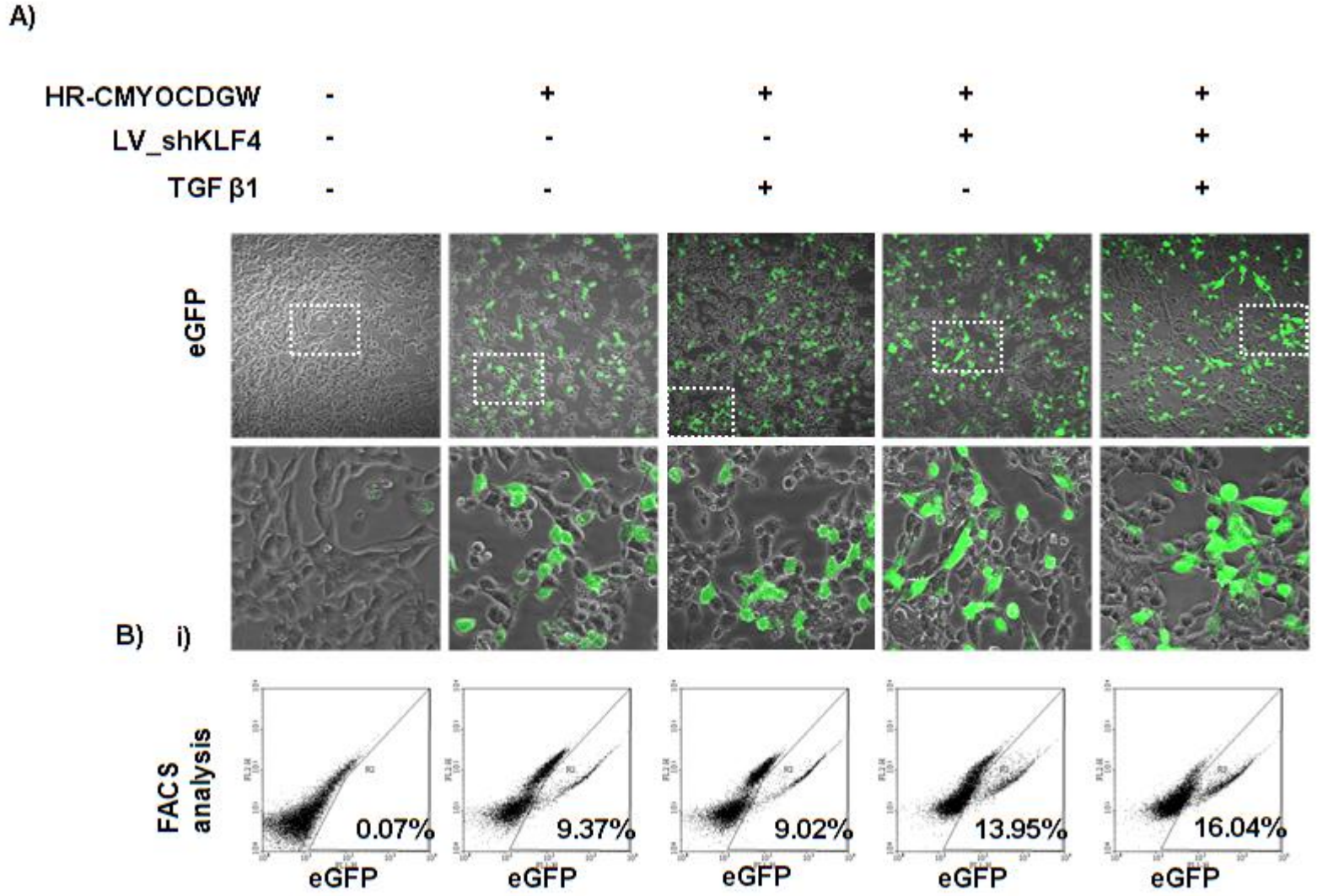


Figure 4-7. Knockdown of KLF4 expression using shKLF4 B lentiviral construct in hSMSPC cells.

To investigate the effect of designed target shKLF4 lentiviral constructs, hSMSPC cells were infected with five different shKLF4 lentiviral supernatant or with empty viral construct as negative control. Next day, the transduced cells were subjected to puromycin selection (1 µg/ml) for 3 days. Total RNA was isolated and the mRNA expression of KLF4 was measured by RTPCR (A) normalised to GAPDH expression, the protein level of KLF4 was estimated by western blot (B&C) and normalised relative to PCNA protein expression. Error bars represent the mean ± SD of three independent experiments**.** n=3, *P<0.05


A)
HR-CMYOCDGW - + + + +
LV_shKLF4 - - - + +
TGF β1 - - + - +
eGFP
B) i)
FACS analysis
0.07%
9.37%
9.02%
13.95%
16.04%
eGFP
eGFP
eGFP
eGFP
eGFP

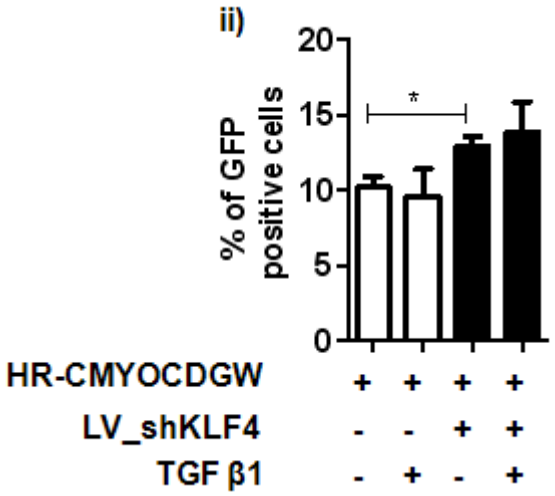


Figure 4-8. Suppression of KLF4 is required for human myocardin promoter expression in hSMSPC.

To evaluate the effect of KLF4 knockdown on human myocardin expression upon TGF β1 stimulation, hSMSPC cells were infected with human myocardin promoter expressing eGFP lentiviral constructs alone (HR-ChMYOCDGW) or with LV_shKLF4 B lentiviral supernatant and stimulated with or without TGF-β1 (5ng/ml) in EGM2 media for 4 days. Confocal microscopy (A) and FACS analysis (B) was used to detect the eGFP expression. The boxed area within the image (A) was shown in higher magnification in bottom panel (A) .i) Representative eGFP FACS analysis showing untransduced and transduced hSMSPC cells. The boxed area within the top panel image (A) was shown in higher magnification in bottom panel. ii) FACS analysis was used to estimate the percentage of eGFP expressing hSMSPC cells. Data are representative of three independent experiments. n=3, *P<0.05.

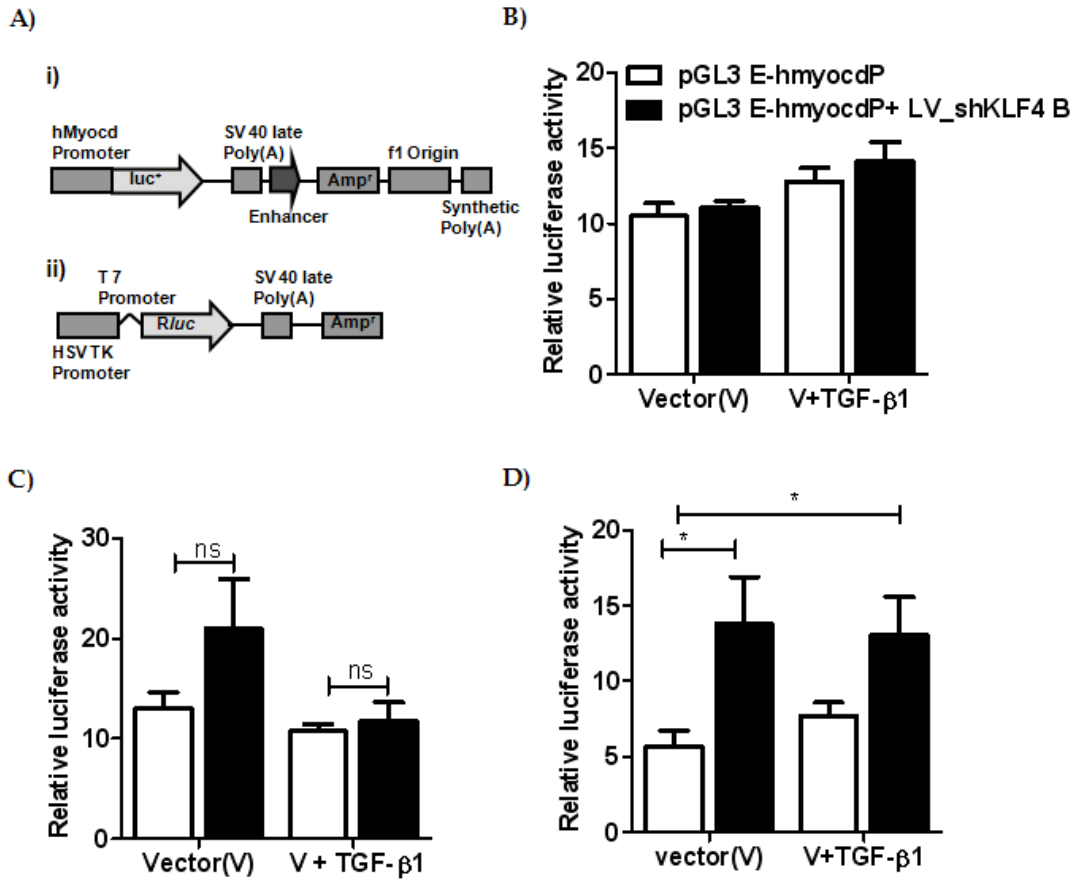


Figure 4-9. Knockdown of KLF4 induced human myocardin promoter activity in time dependent manner detected by luciferase reporter assay.

A) Schematic representation of luciferase reporter plasmid. i) Schematic representation of pGL3 E plasmid containing human myocardin promoter driving firefly luciferase reporter gene. ii) Schematic representation of pRL-TK plasmid B) to measure the time course effect of TGF-β1 on human myocardin promoter activity in hSMSPC and KLF4 knockdown hSMSPC cells, the cells were infected with LV_shKLF4 B or empty vector lentiviral supernatant. At 24 h post transduction, cells were cotransfected with pGL3 E containing the human myocardin promoter driving the expression of firefly luciferase reporter construct and pRL-TK Renilla luciferase reporter construct. At 16 h post transfection the cell were stimulated with or without TGF-β1 (5ng/ml). Dual luciferase activity were measured at 24 (B), 48 (C) and 72 (D) hours post stimulation and human myocardin promoter activity was defined as ratio of firefly luciferase activity/Renilla luciferase activity. The black bar represents human myocardin promoter directed luciferase activity in hSMSPC cells and the white bar represents human myocardin promoter directed luciferase activity in KLF4 knockdown hSMSPC cells. Results are expressed as means ± SD of three independent experiments n=3, *P<0.05

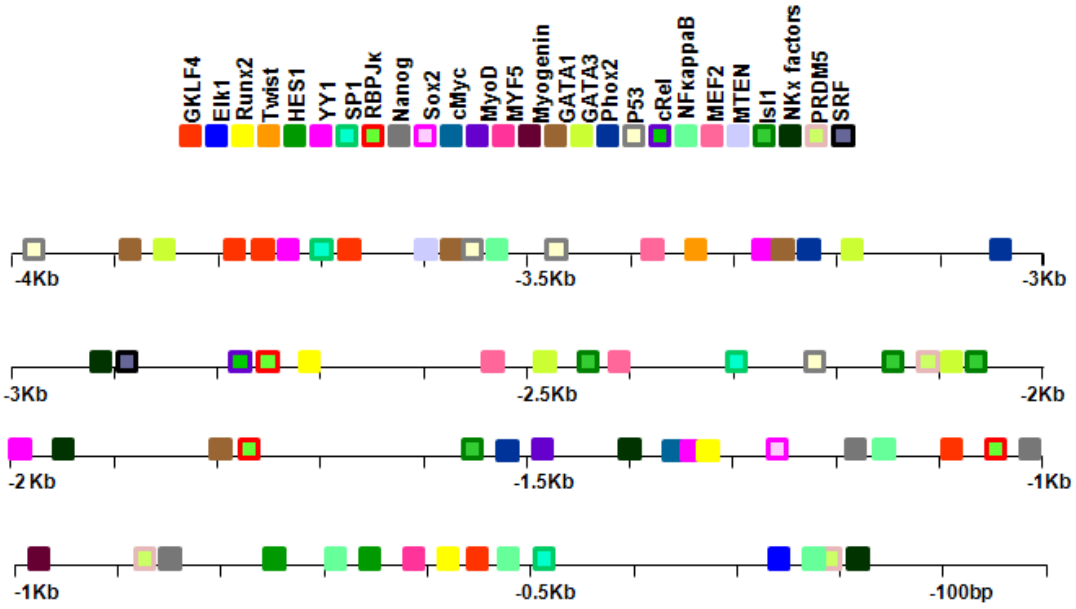
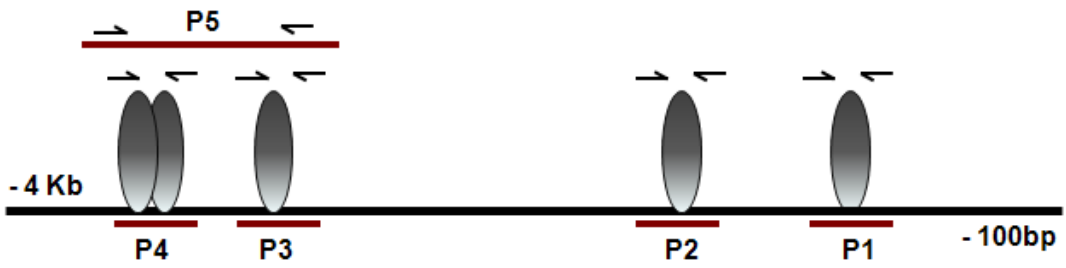


Figure 4-10. Schematic showing putative binding sites for myocardin regulating transcription factors within the 3.925 Kb cloned human myocardin promoter.

GKLF indicates GKLF4 - gut-enriched Krüppel-like factor , Elk1- ETS domain-containing protein, Runx2 - Runt related transcription factor 2, Twist1 - Twist-related protein 1, HES - Hairy and enhancer of split-1,YY1 - Yin Yang 1, SP1- specificity protein 1, RBPjk - Recombining binding protein suppressor of hairless, Sox2 - SRY (sex determining region Y)-box 2, MYF5 - Myogenic factor 5, Phox2 - Paired mesoderm homeobox protein 2, MEF2 - myocyte enhancer factor-2, PTEN - Phosphatase and tensin homolog, Isl-1 - Insulin gene enhancer protein, PRDM5 - PR domain containing 5, SRF - serum response factor.

A)



B)

Primer Name	Primer Sequence	Primer position	Amplicon size
P1_KLF4_S1_F	GGAGATCCCGGCCGCTTCCCTTTCG	-705/-681	195 bp
P1_KLF4_S1_R	ATCTGGGAGAGGGGCGCGAGCTAAA	-535 /-511	
P2_KLF4_S2_F	ACTCTGGGTCGGTTACGGAATGGAT	-1207/-1183	184 bp
P2_KLF4_S2_R	CCGAGGGAATGGGAAAAGATACCTG	-1024/-1148	
P3_KLF4_S3_F	GCCAAAATGAAGGGGAAAGGGACAG	-3776/-3752	182 bp
P3_KLF4_S3_R	GAACTCCTGCGCTCAGGTGATCTGC	-3619/-3595	
P4_KLF4_S4_F	ATCCATGCCTGGACTCCTGACCATG	-3892/-3868	198 bp
P4_KLF4_S4_R	CTTTTTACTTCCCCTGCCCCAACCA	-3719/-3695	
P5_KLF4_S3_S4_F	ATCCATGCCTGGACTCCTGACCATG	-3892/-3868	298 bp
P5_KLF4_S3_S4_R	GAACTCCTGCGCTCAGGTGATCTGC	-3619/-3595	

Figure 4-11. Schematic representation showing ChIP-PCR amplification of KLF4 binding region within the myocardin promoter.

A) Graphical representation of upstream region of human myocardin promoter displaying location of putative KLF4 binding site, S1 to S5 (grey elongated circles) and their corresponding ChIP-RT-PCR amplicon. The ChIP-PCR amplicon were shown by the brown bars below the KLF4 putative binding sites, labeled P1 to P5. The forward and reverse arrow indicates position of forward and reverse primers used in ChIP assay. B) The primer sequence used in RT- PCR amplification and their relative position on human myocardin promoter was shown as table.

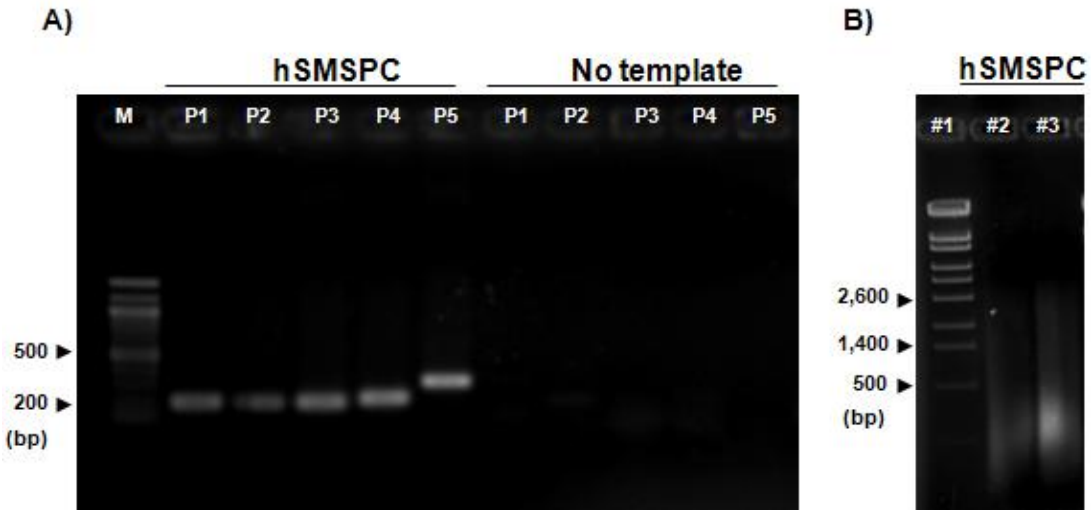


Figure 4-12. Checking qRT-PCR designed primers for amplification of predicted KLF4 binding sites and optimization of sonication in hSMSPC cells.

A) PCR amplification of ChIP assay primers in hSMSPC. To check the specificity of designed PCR primers sets (listed on table Fig.4.11B) flanking potential KLF4 binding sites, PCR amplification was performed for 30 cycles using the genomic DNA from hSMSPC to ensure that the designed primers produce single amplicon from myocardin promoter region and analysed on a 1% TAE agarose gel stained with ethidium bromide. M – 100 bp DNA ladder, P1, P2, P3, P4 are primer set flanking site 1, 2, 3 and 4 respectively. P5 – primer set flanking site 3 & 4. B) Agarose gel analysis of sheared DNA from hSMSPC after sonication, showing the optimal size of fragmentation (200 to 1500 bp) visualized by ethidium bromide. Following the method outlined in section 4.2.13, sonicated chromatin was prepared from formaldehyde cross linked hSMSPC cells and, the degree of chromatin shearing was analysed by loading 1 µg and 2 µg of sheared DNA on 1% TAE agarose gel, visualized with ethidium bromide. Lane #1 - 1 Kb DNA ladder, Lane #2 and #3 are 1 µg and 2 µg of hSMSPC sonicated and purified DNA. The sonicated chromatin for 6 pulses yielded optimal sheared chromatin ranging between 200 bp to 1500 bp suitable for ChIP assay.

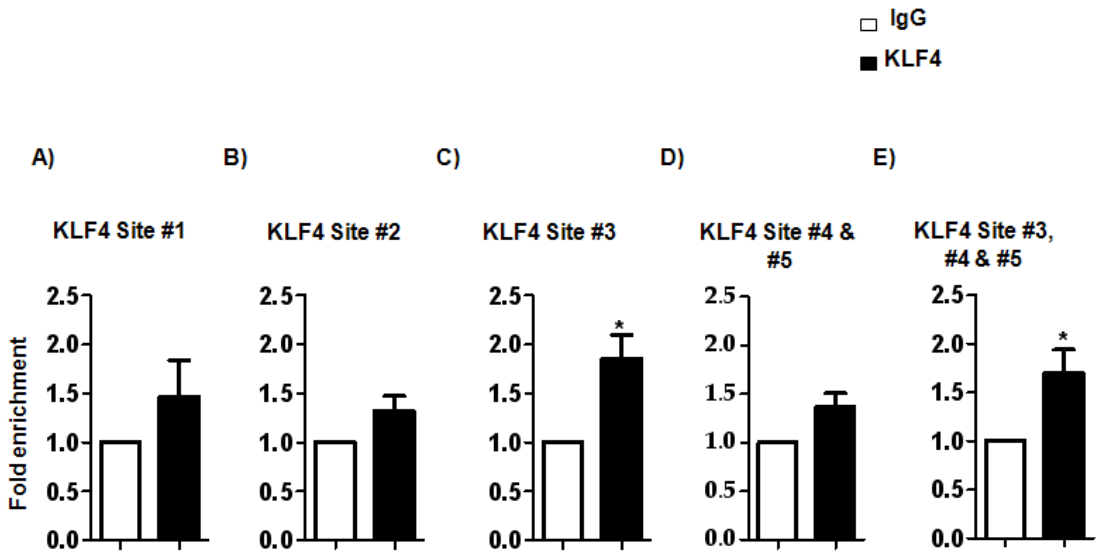


Figure 4-13. Binding of KLF4 to a human myocardin promoter at site 3 (-3702) in hSMSPC cells.

To examine whether KLF4 binds to myocardin promoter region, qRT- PCR was performed on KLF4 immunoprecipitated DNA in hSMSPC cells. The cells ($8x10^6$) were fixed with 1% formaldehyde and the DNA was sheared by sonication. ChIP assay was performed on sheared chromatin from hSMSPC using antibody against KLF4 or normal IgG. The chromatin immunoprecipitated DNA was extracted by reversal of cross links followed by proteinase K and RNase A treatments. The recovered chromatin through ChIP assay was analysed by 40 cycles of qRT-PCR using the primers flanking the KLF4 binding sites (KLF4-Site#1 to KLF4-Site#5) listed in table Fig.4.11.B. The results were presented as fold enrichment over IgG control (A-E). The results showed that ChIP assay performed with KLF4 antibody and PCR with primer flanking site 3 (C) greatly enriched for human myocardin promoter DNA in hSMSPC cells. Values are the means ± SD of three independent experiments. n=3, *P<0.05.

4.4. Discussion

These experiments aimed to identify the major transcription factors involved in the regulation of human myocardin gene expression. The current study hypothesized that the presence of the transcriptional repressor KLF4 may regulate human myocardin promoter activity and block hSMSPC differentiation into mature contractile SMCs. The results from the present study demonstrate that 1) Myocardin promoter activity is restricted to HASMC and hSMSPC. 2) Human myocardin promoter has three ECR between human, rat and mouse and has putative binding sites for many transcriptional repressors including KLF4. 3) KLF4 has five evolutionary conserved putative binding sites within the human myocardin promoter. 4) KLF4 silencing resulted in increased human myocardin promoter activity in hSMSPC. 5) KLF4 binds within the human myocardin promoter predominantly at site 3 (-3702) position upstream of TSS in cultured hSMSPC cells.

Molecular mechanisms involved in transcriptional regulation of SMC are complex involving unique combinations of transcription factors and cytokine stimulation. Myocardin is a strong transcriptional coactivator of smooth muscle gene expression and regulated by many transcriptional modulators and signaling pathways [105, 164]. However, the transcriptional regulation of myocardin expression itself during smooth muscle differentiation is not fully elucidated. The current study specifically identifies a critical role for KLF4 in the transcriptional regulation of the myocardin promoter. Furthermore, a significant advance in this study is the identification of five putative KLF4 binding sites within the human myocardin promoter. KLF4 has been widely reported to be rapidly upregulated in proliferative SMCs upon vascular injury. KLF4 abrogates myocardin induced activation of SMC markers via hypoacetylation of H4 histones [138]. Therefore, KLF4 appears to play a major role in regulation of myocardin induced transactivation of smooth muscle marker gene expression. In this study, following knockdown of KLF4 myocardin promoter driven gene expression was significantly increased, suggesting that presence of abundant KLF4 might act as repressor for human myocardin promoter activity in hSMSPC cells. The current study provides first line evidence that KLF4 represses human myocardin promoter activity through binding on its promoter at -3702 upstream of TSS in hSMSPC cells. Taken together, these experiments indicates for the first time a novel repression mechanism of KLF4, through binding within the myocardin promoter and regulating myocardin gene expression in hSMSPC cells.

TGF-β1 has been shown to regulate the expression of KLF4. TGF-β1 is demonstrated to induce myocardin expression in SMC differentiation of progenitor cells such as multipotent SMC progenitor's 10T1/2 cells [240] and neural crest cells [223]. Davis-Dusenberry *et al* [211]., demonstrate that TGF-β1 downregulates KLF4 expression through induction of myocardin and miR 143/145 in SMCs. They further showed that silencing of KLF4 using siRNA induced myocardin mRNA expression in SMCs, suggesting that repression of KLF4 by TGF-β1 is critical for the induction of SM contractile phenotype in SMCs. Consistent with this findings, the current study demonstrated that silencing of KLF4 followed by TGF-β1 stimulation induced the human myocardin promoter activity in hSMSPC cells. However, a small delay in TGF-β1 induced myocardin promoter activation was observed in hSMSPC cells. This delay might be due to suppression of myocardin by Smad 3 in the initial stages TGF-β1 induced progenitors' cells differentiation into SMC [347].

Recently, three closely related KLF transcription factors, KLF2, KLF4 and KLF5 have been identified as key regulators in controlling SMC development and phenotypic modulation [139, 348]. It has been reported that KLF4 and KLF5 bind to TCE elements within the SM α actin and SM 22α promoters. KLF4 repress TGF-β1 induced SM 22α promoter whereas KLF5 activates TGF-β1 dependent activation of the same promoter, indicating that KLF4 and KLF5 can exhibit opposing effect on the promoter [139]. Thus, it would be interesting to expand the current study to explore the possibility of other KLF transcription factors like KLF2 and KLF5 binding to the putative KLF4 binding sites within the human myocardin promoter in hSMSPC cells.

The ChIP analysis in hSMSPCs showed increase in enrichment of KLF4 at all five putative binding sites within the human myocardin promoter. However, more significant enrichment was observed within the site 3 (-3702 bp) of the human myocardin promoter. These initial studies indicate the critical role of KLF4 in regulation of myocardin gene expression in hSMSPCs. However, further analysis using site directed mutation analysis and EMSAs are warranted to fully establish the possible functionality of each site.

Following vascular injury *in vivo* and PDGF-BB in cultured cells has been reported to induce KLF4 expression with downregulation of SM markers in SMCs [349]. The ChIP analysis in the current experiment was carried out in unstimulated hSMSPC cells. Hence, it would also be interesting to study the effect of PDGF-BB stimulation on hSMSPC for the gain of KLF4 function and knockdown KLF4 for loss of function to examine the occupancy of these putative KLF4 binding sites within the

human myocardin promoter in hSMSPC cells. Such findings would not only shed light on the mechanism of SMC differentiation but also identify KLF4 as a therapeutic target for controlling SMC dedifferentiation.

In summary, the present study for the first time identified putative binding sites for KLF4 and demonstrated direct binding of KLF4 within the human myocardin promoter predominantly at position -3702 bp using ChIP assay in hSMSPC cells. The following study will explore whether shRNA mediated silencing of endogenous KLF4 or overexpression of myocardin in differentiation media can induce SMC differentiation in hSMSPC cells.

5. Specific Aim 3 - Determination of molecular mechanism underlying myocardin mediated smooth muscle differentiation in hSMSPCs

5.1. Introduction

Myocardin is a strong coactivator for SRF-CArG dependent transactivation of smooth muscle gene transcription in smooth muscle cells and cardiomyocytes [94, 142, 143, 145]. Myocardin physically interacts with SRF and activates a set of contractile smooth muscle genes in SMC [350]. Overexpression of myocardin alone is sufficient to induce SMC differentiation markers such as calponin and SMMHC in 10T1/2, NIH 3T3 and 3T3 L1 cells [350]. Ectopic expression of myocardin in xenopus embryo activates the expression of cardiac specific and smooth muscle genes detected by in situ hybridization [351]. In stem cells, forced expression of myocardin in ESC induced SMC differentiation with upregulation of SMC markers such as SM α actin, calponin and SM22α [145]. In contrast, gene transfer of a dominant negative form of myocardin and siRNA mediated knockdown decreased the expression of SMC marker genes in SMC [145]. In addition, downregulation of myocardin expression in xenopus embryo using antisense morpholino technique results in repression of cardiac marker expression [351].

Significantly, the expression of myocardin gets downregulated in parallel with SMC differentiation marker genes in response to vascular injury [98] as well as in cultured SMCs [143]. Myocardin null mutant mice survive only to embryonic day E10.5 and show abnormalities in vascular development specific to SMC differentiation [158]. Genetic analysis of mice harboring a selective ablated myocardin gene in neural crest cell derived SMC died prior to postnatal day P3 with patent ductus arteriosus (PDA) and exhibit cell autonomous defect in SMC contractile proteins [352]. Taken together, these studies suggest that myocardin is sufficient and necessary to induce SMC differentiation and contractile SMC phenotype in muscle and non muscle cells.

TGF-β1 signaling plays a crucial role in SMC development and differentiation [241, 353]. Gene targeting approaches to TGF-β1 function in mice have shown that loss of TGF-β1 signaling results in dysregulated SMC differentiation and disruption of vessel wall integrity [354]. Furthermore, deletion of one allele of TGF-β1 in young mice resulted in downregulation of SMC differentiation markers [355]. These studies show a tight relationship between TGF-β1 signaling and SMC differentiation during embryonic development.

Several *in vitro* models have been employed to study the molecular mechanism underlying the TGF-β1 induced SMC differentiation in progenitor cells [239, 259]. TGF-β1 has been reported to induce SMC differentiation in various cell types including neural crest stem cells [239], mesenchymal progenitor cells [240]

and endothelial cells [266]. TGF-β1 also induces the SM differentiation markers in cultured SMCs [237]; these studies demonstrate the important role of TGF-β1 in progenitor cell differentiation into SMC. Transforming growth factor control element (TCE), *cis* element located within SM promoter regions was found to be highly evolutionally conserved across species and reported to be essential for TGF-β1 inducibility of SMC differentiation markers in SMC and fibroblast [237, 356]. Of note, mutation of the SM α actin promoter TCE element completely attenuated TGF-β1 induced SM marker activation in SMCs [139, 140].

Multiple signaling pathways involved in TGF-β1 induced transactivation of SM differentiation marker gene transcription [357]. The molecular mechanism of TGF-β1 mediated activation of SMC marker proteins transcription reveals three TGF-β1 responsive elements within SM promoters such as the CArG box, SBE and TCE element [213].

In TGF-β1 induced SMC differentiation, Smad3, the major TGF-β1 mediator, has been reported to bind to one of three TGF-β1 responsive elements within SM promoters depending on cell type. Smad 3 binds to SRF and regulates CArG-SRF dependent SM22α transcription during myoblast differentiation of 10T1/2 cells [358] whereas Smad3 binds to SBE element and activates SM22α gene transcription in monc-1 [239] . Smad3 has also been shown to interact with myocardin to synergistically activate SM22α activity in 10T1/2 cells [213]. These studies suggest that myocardin and TGF-β1 signaling are essential for SMC differentiation.

KLF4, a zinc finger transcription factor, has been identified as a binding factor that can bind to TCE regions located upstream within smooth muscle promoters and downregulates expression of smooth muscle marker genes [140]. Of interest, overexpression of KLF4 protein repressed TGF-β1 induced SMC marker activation in 101/2 cells [140] and also KLF4 attenuates myocardin induced activation of SMC markers [167]. However, inhibition of endogenous KLF4 using antisense morpholino restores SM α actin and SMMHC expression [140]. KLF4 mediates PDGF-BB induced transcriptional suppression of smooth muscle differentiation marker genes [167]. Furthermore, siRNA mediated knockdown of KLF4 expression partially abrogates PDGF-BB induced downregulation of SM markers in cultured SMCs [167]. These studies strongly suggest that KLF4 acts as a potent transcriptional repressor for smooth muscle differentiation markers.

Retroviral expression of KLF4 along with Oct4, Sox2 c-myc have the ability to induce reprogramming of human adult fibroblasts to become embryonic stem cell-

like cells termed as iPS induced pluripotent stem cells. Thus, KLF4 is a key transcription factor involved in the self renewal and pluripotency maintenance of the stem cells.

Results from previous studies demonstrate that the hSMSPC cells have low levels of myocardin and higher levels of KLF4 expression as measured by qRT-PCR. Of significance, genomatrix analysis of 3.9 Kb (-4007 to -100 bp) of the human myocardin promoter region for putative transcription binding sites reported five putative binding sites for KLF4 within the human myocardin promoter and results from ChIP assay further verified binding of KLF4 within the myocardin promoter region. Moreover, previous findings demonstrated that TGF-β1 can induce the human myocardin promoter directed luciferase activity in KLF4 knockdown cells.

Therefore, this study **hypothesis that TGF-β1 can induce SMC differentiation in KLF4 knockdown or myocardin overexpressed hSMSPC cells (Fig.5-1).**

5.2. Methods

5.2.1. Cell culture and KLF 4 detection by Western blotting

To compare KLF4 expression levels between HASMC and hSMSPC cells, HASMC and hSMSPC were grown to confluence in and EGM2 respectively was harvested in ice cold PBS. Then, western blot was performed on harvested cells following the protocol outlined in general methods 2.14 with anti-KLF4 antibody and rabbit HRP-labelled secondary antibody

5.2.2. KLF4 immunofluorescence analysis

For KLF4 immunofluorescence, hSMSPC and HASMC cells were seeded on nunc wells at 20,000 cells per cm^2 and allowed to grown for 24 hours. The cells were harvested, fixed and immunostained with anti-KLF4 antibody (1:200) as described in general methods 2.7.

5.2.3. LV_shKLF4 mediated knockdown of KLF4 protein and hSMSPC differentiation

To differentiate hSMSPC into SMC, hSMSPC cells were seeded at 50,000 cells per cm^2 plating density on nunc wells coated with 5µg/cm^2 fibronectin (Sigma, St.Louis, MO) in EGM 2 media a day before the transduction. The cells were allowed to settle down and LV_shKLF4 B or empty vector lentivirus supernatant diluted in differentiation media was added to the cells. Twenty four hours of

transduction, the cells were cultured in differentiation media containing 1µg/ml puromycin in order to remove the non transduced cells. The differentiation of hSMSPC into SMC was assessed at day 7 post stimulation by immunofluorescence as outlined in general methods 2.7 with smooth muscle markers antibodies such as SM α actin (1 in 200), Calponin (1 in 200) and SMMHC (1 in 200).

5.2.4. Cell culture and *in vitro* hSMSPC differentiation assay

hSMSPC and HeK 293T (negative control) cells were maintained in EGM2 and DMEM 10% media respectively. To examine whether lentivirus mediated myocardin gene expression can induce SMC differentiation of hSMSPC cells, both hSMSPC and HeK 293T cells (used as negative control) were plated at 50,000 cells per cm^2 on nunc wells. The cells were allowed to settle down and transduced with myocardin lentivirus (LV_myocardin) at 5 MOI in EGM2 media. The myocardin lentiviral vector plasmid (pLV-CMV-myocL-HA) [359] encoding full – length human myocardin cDNA was the generous gift of Antoine A.F. de Vries from Leiden University Medical Center, the Netherlands. The lentiviral particles were produced using the protocol outline in general methods 2.9. Then, media was changed to SmGM2 with 1% FBS or differentiation media after 24 hours post transduction. To measure the time dependent SMC differentiation over 14 days, the lentiviral transduced cells were assessed for SMC differentiation at different time points day 0, day 3, day 7 and day 14 of TGF-β1 stimulation using immunofluorescence protocol outline in general methods section 2.7. Smooth muscle marker antibody such as SM α actin (1 in 200), Calponin (1 in 200) and SMMHC (1 in 200) and HA-tag (1 in 200) were used to measure the SMC differentiation in hSMSPC and HeK 293T cells.

To quantify the SMC marker expression, numbers of SMC marker expressing cells were counted in 10 random fields from three separate experiments. The percentage of SMC positive cells and myofilamentous cells were determined by dividing the SMC positive cells by the total number of DAPI positive cells.

5.2.5. Smooth muscle marker detection by Western blotting

To quantify the smooth muscle protein expression in differentiated hSMSPC cells, the hSMSPC cells were plated at 50,000 cells per cm^2 in 6 well plate and transduced with myocardin lentivirus at 5 MOI in EGM2 media. The media was changed to SmGM2 with 1 % FBS after 24 hours post transduction and maintained in SmGM2 1% FBS media for 7 days. To measure the SMC proteins overtime the protein was extracted from transduced hSMSPC cells day 0, day 1, day 2, day 3,

day 7 and day 14 and analysed by western blot by following protocol outlined in section 2.14, for smooth muscle protein antibodies such as SM α actin (1 in 500), Calponin (1 in 500). β lamin (1 in 500) was used as loading control and the bands were quantified relative to PCNA using Image J software.

5.3. Results

5.3.1. hSMSPC has high KLF4 expression level as compared to HASMC

To quantify KLF4 protein expression levels, hSMSPC and HASMC cells were grown in chamber slides and immunostained using anti-KLF4 antibody. The results showed that hSMSPC displayed high levels of KLF4 expression when compared to HASMC (Fig.5.2.A). A strong KLF4 expression, mostly accumulated in the corner of the nucleus was noted in hSMSPCs whereas a diffused nuclear staining was observed in hSMC. Western blot analysis was also performed with hSMSPC and hSMC and detected the presence of KLF4 protein as doublet band around 55 K Da in both cell types. The reason for KLF4 doublet band is unknown. KLF4 expression was normalised to β-actin. Consistent with immunofluorescence analysis results, hSMSPC showed elevated KLF4 expression as compared to hSMC as shown in Fig.5.2.B&C.

5.3.2. Silencing of KLF4 results in increased SMC differentiation of hSMSPCs

To address the role of KLF4 in hSMSPC differentiation, we examined the effect of KLF4 knockdown on hSMSPC differentiation using shRNA lentivirus vectors. hSMSPC cells were transduced with LV_shKLF4 B or empty vector and stimulated with TGF-β1 for 7 days. By four days after TGF-β1 stimulation, KLF4 knockdown hSMSPC cells revealed a marked increase in cell size (Fig.5.3) compared to those transduced with an empty vector measured by phase contrast microscopy. Immunostaining for smooth muscle markers such as SM α actin and calponin revealed that KLF4 knockdown accelerated SMC differentiation in hSMSPC (Fig.5.4.A&B).

In KLF4 knockdown hSMSPC cells, both the expression of SM α actin and calponin was significantly increased by TGF-β1 stimulation during SMC differentiation to 9 fold and 23 fold with 17% and 8% of myofilament, respectively, after 7 days of stimulation as compared with empty vector shown in Fig. 5.4.A&B.

These results indicate that TGF-β1 stimulation induced SMC differentiation in KLF4 knockdown hSMSPC cells.

5.3.3. Overexpression of myocardin induced smooth muscle differentiation in hSMSPC cells

To examine whether overexpression of myocardin can induce SMC differentiation specifically in hSMSPC cells, undifferentiated hSMSPC cells or HeK 293T (negative control) cells were infected with a lentiviral vector encoding myocardin cDNA driving a HA-tag in EGM2 media. At 24 hours post transduction, the media was replaced by SmGM2 1% FBS or differentiation media and maintained for a period of 14 days. The cells were collected at day 0, 1, 2, 3, 7 and 14 time points and analysed by SDS PAGE as described in methods for SMC markers such as SM α actin and calponin. Protein levels were normalised to β lamin and to the control (day 0 untransduced cells). By Western blotting, SM α actin, calponin and β lamin proteins were detected at position 42 kDa, 34 kDa and 55 kDa respectively in lysate of both hSMSPC and HeK 293T cells. Significantly, in hSMSPC cells over expression of myocardin resulted in upregulation of smooth muscle marker expression such as SM α actin and calponin in time dependent manner (Fig. 5.5 (A) & (B)). The expression of SM α actin (Fig 5.5 (C)) was increased 25 fold as compared to HeK 293T cells with 15 fold increases after 14 days of transduction (Fig. 5.5 (D)) The expression of calponin in hSMSPC was significantly increased to 18 fold at day 2 (Fig. 5.5 (E)) and reached a maximum of 62 fold at day 14 of transduction whereas calponin was less efficiently induced only 7.4 fold at day 14 in HeK 293T cells (Fig. 5.5 (F). These results demonstrate that over expression of myocardin induced SMC differentiation in hSMSPC but not in HeK 293T cells.

The levels of myofilament organisation were compared between differentiated hSMSPC and HeK 293T cells post TGF-β1 stimulation using immunofluorescence analysis. At day 0, the early smooth muscle marker protein, SM α actin showed a punctate staining pattern within the nucleus detected by immunostaining in both hSMSPC and HeK 293T cells (Fig.5.6 (A)). However, the late SMC lineage genes such as calponin and SMMHC marker expression were undetectable in both undifferentiated hSMSPC (Fig.5.6 (A)) and HeK 293T cells (Fig.5.8 (A)).

The expression of SM α actin began to increase from day 3 onwards and gradually spreads throughout the cells with myofilament organisation in both myocardin transduced hSMSPC alone or in combination with TGF-β1 as compared to undifferentiated hSMSPC cells (Fig.5.6 (B & C). The results were shown in table

5-2. These results suggest that TGF-β1 has similar effect on SM α actin expression but TGF-β1 significantly increased the myofilamentous SM α actin positive cells to 23% and 52% as compared to LV_myocardin alone with 14% and 27% (Fig.5.6.(B&C).) after day 7 and day 14 respectively. Similarly, LV_myocardin transduction alone or in combination with TGF-β1 treatment of HeK 293T cells induced SM α actin expression in time dependent manner to a lesser extent. The expression of SM α actin in LV_myocardin treatment alone and with TGF-β1 was shown in table 5-1. However, both LV_myocardin alone and with TGF-β1 failed to induce myofilament organisation in HeK 293T cells (Fig.5.8 A-C).

The expression of both calponin and SMMHC was increased to a similar extent in both LV_myocardin transduced alone or in combination with TGFβ1 stimulated hSMSPC cells (Fig.5.7 A-C). However, TGF-β1 stimulation increased both calponin and SMMHC myofilament positive cells to a lesser extent as compared to LV_myocardin transduced hSMSPC cells (Fig.5.7 A-C). The results were shown in table 5-1.

In contrast, myocardin induced both calponin and SMMHC less efficiently in LV_myocardin transduced alone or LV_myocardin with TGF-β1 stimulated HeK 293T cells. However, both LV_myocardin alone and with TGF-β1 failed to induce neither calponin and SMMHC myofilament organisation in HeK 293T cells (Fig.5.9 A-C). The results were shown in table 5-2.

Taken together, these data demonstrates that overexpression of myocardin activates SMC differentiation with expression of SMC lineage genes such as SM α actin, calponin and SMMHC more efficiently in hSMSPC cells than in HeK 293T cells.

Table 5-1.The results of the smooth muscle marker expression and myofilament organisation in differentiated hSMSPC cells stimulated with or without TGF- β1.

	hSMSPC			hSMSPC cells with TGF-β1		
	Day 3	**Day 7**	**Day 14**	**Day 3**	**Day 7**	**Day 14**
SM α actin	16%	46%	81%	16%	39%	79%
SM α actin myofilament organisation	-	14%	27%	-	23%	52%
Calponin	87%	90%	92%	87%	91%	88%
Calponin myofilament organisation	21%	66%	69%	32%	78%	71%
SMMHC	49%,	68%,	91%	50%,	60%,	90%
SMMHC myofilament organisation	7%	22%	58%	17%	27%	62%

Table 5-2. The results of the smooth muscle markers expression and myofilament organisation in differentiated HeK 293T cells stimulated with or without TGF- β1.

	HeK 293T			HeK 293T cells with TGF-β1		
	Day 3	**Day 7**	**Day 14**	**Day 3**	**Day 7**	**Day 14**
SM α actin	8.3%	13.2%	16%	5.6%	12%	22%
SM α actin myofilament organisation	-	-	-	-	-	-
Calponin	3.4%	20%	20%	3.9%	21%	20%
Calponin myofilament organisation	-	-	-	-	-	-
SMMHC	0.5%	11%	12%	2.2%	13%	20%
SMMHC myofilament organisation	-	-	-	-	-	-

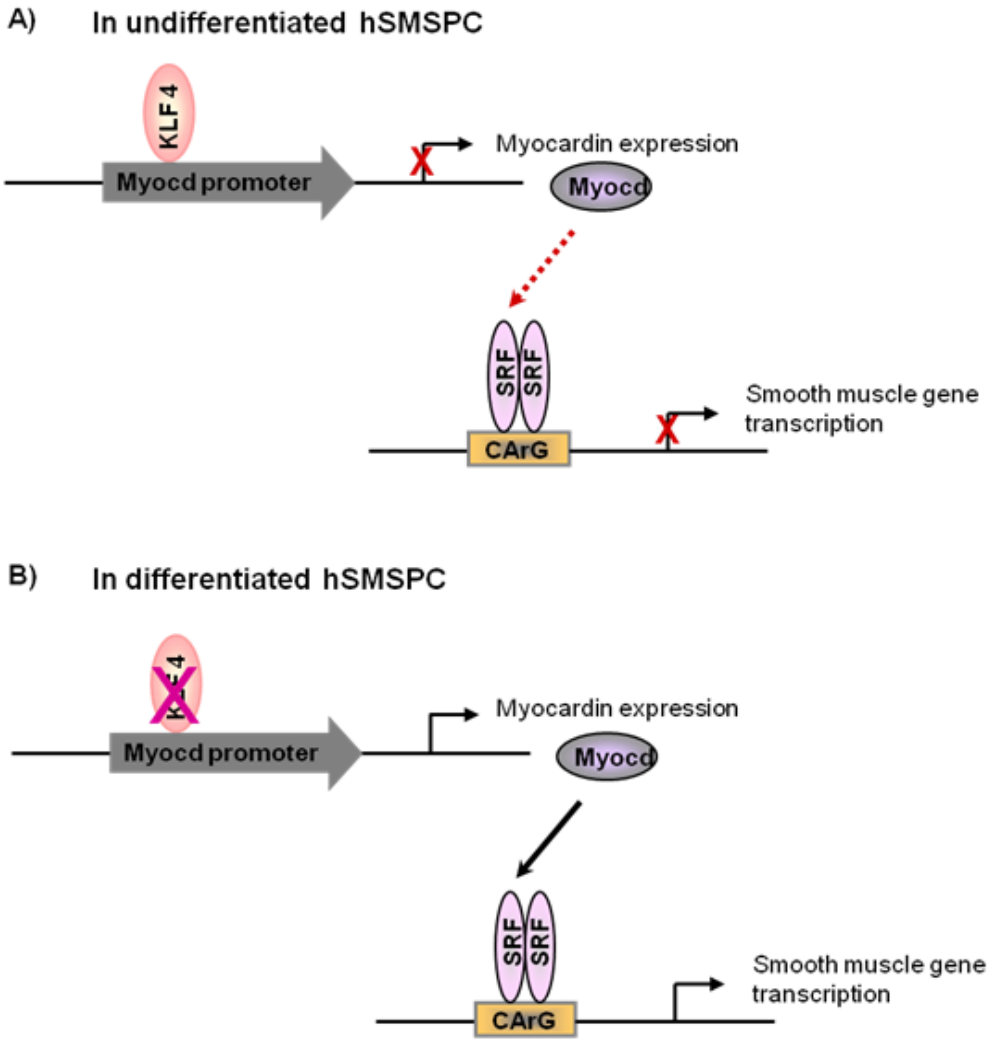


Figure 5-1. Schematic representation showing hypothetical molecular mechanism by which KLF4 inhibits human myocardin gene expression in hSMSPC A) In undifferentiated hSMSPC, KLF4 bind to myocardin promoter and represses myocardin gene transcription which in turn unable to activate SRF-CArG smooth muscle transcription. B) In differentiated hSMSPC, knockdown of KLF4 increases the myocardin expression which transactivates the smooth muscle gene transcription.

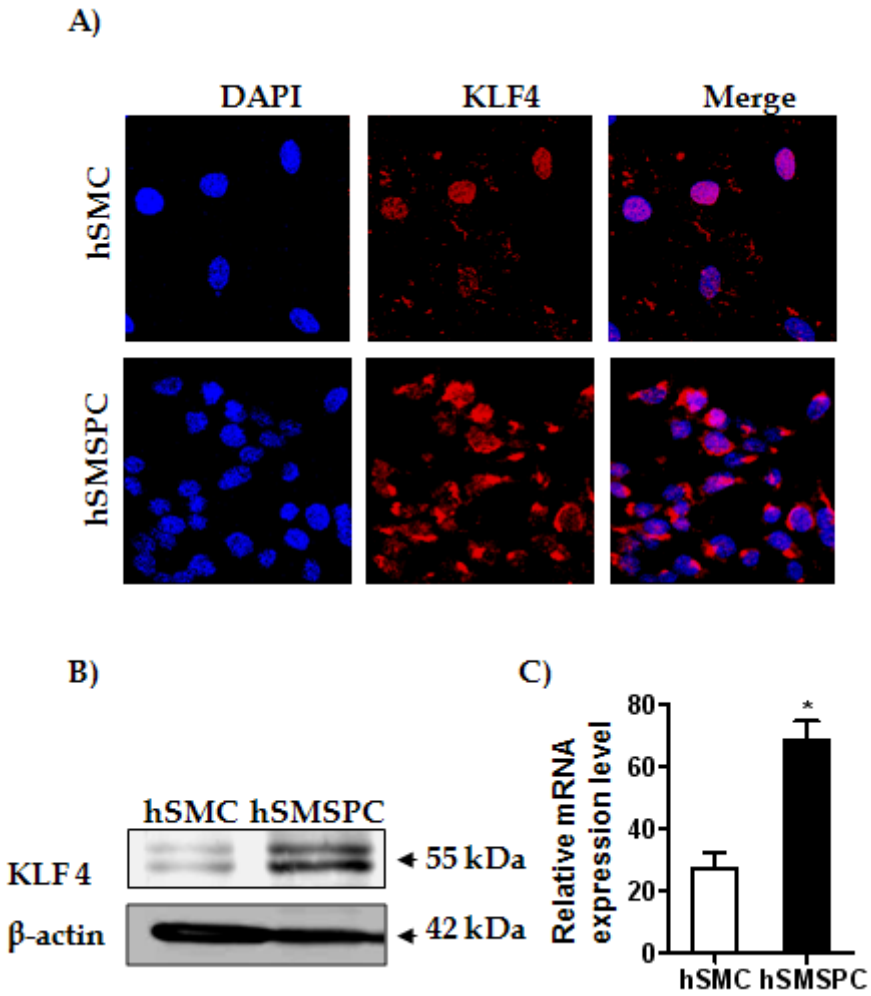


Figure 5-2. Comparison of KLF4 protein expression levels from hSMSPC with HASMC by immunofluorescence and western blot analysis. A) To check the KLF4 expression level, hSMSPC and hSMC were grown on nunc chamber slides and immunostained with KLF4 antibody. KLF4 expression was predominantly nuclear localised in hSMC whereas KLF4 expression was mixed both nuclear and perinuclear in hSMSPC. B & C) Western blot analysis of protein isolated from confluent hSMC (lane 1) and hSMSPC (lane 2) using KLF4 antibody. β actin (42 kDa) was used as loading control. Increased expression of KLF4 as doublet band around 55-KDa was detected in hSMSPC as compared with hSMC cells. Data are representative of three independent experiments. n=3, *P<0.05**.**

A)

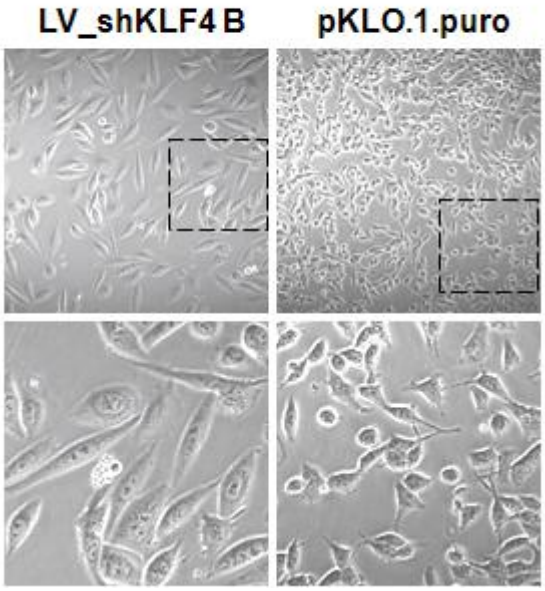


Figure 5-3. Effect of shRNA mediated KLF4 knockdown on cell morphology in hSMSPC. A) hSMSPC cells were infected with LV_shKLF4 B or an empty vector (pKLO.1.puro) in differentiation media and subjected to puromycin after 24 hours post transduction. shRNA mediated knockdown of KLF4 induced morphological changes in hSMSPC after 5 days of puromycin selection as compared to empty vector measured using phase contrast light microscope. The lower panel represents an enlarged view of black boxed area from respective top panel.

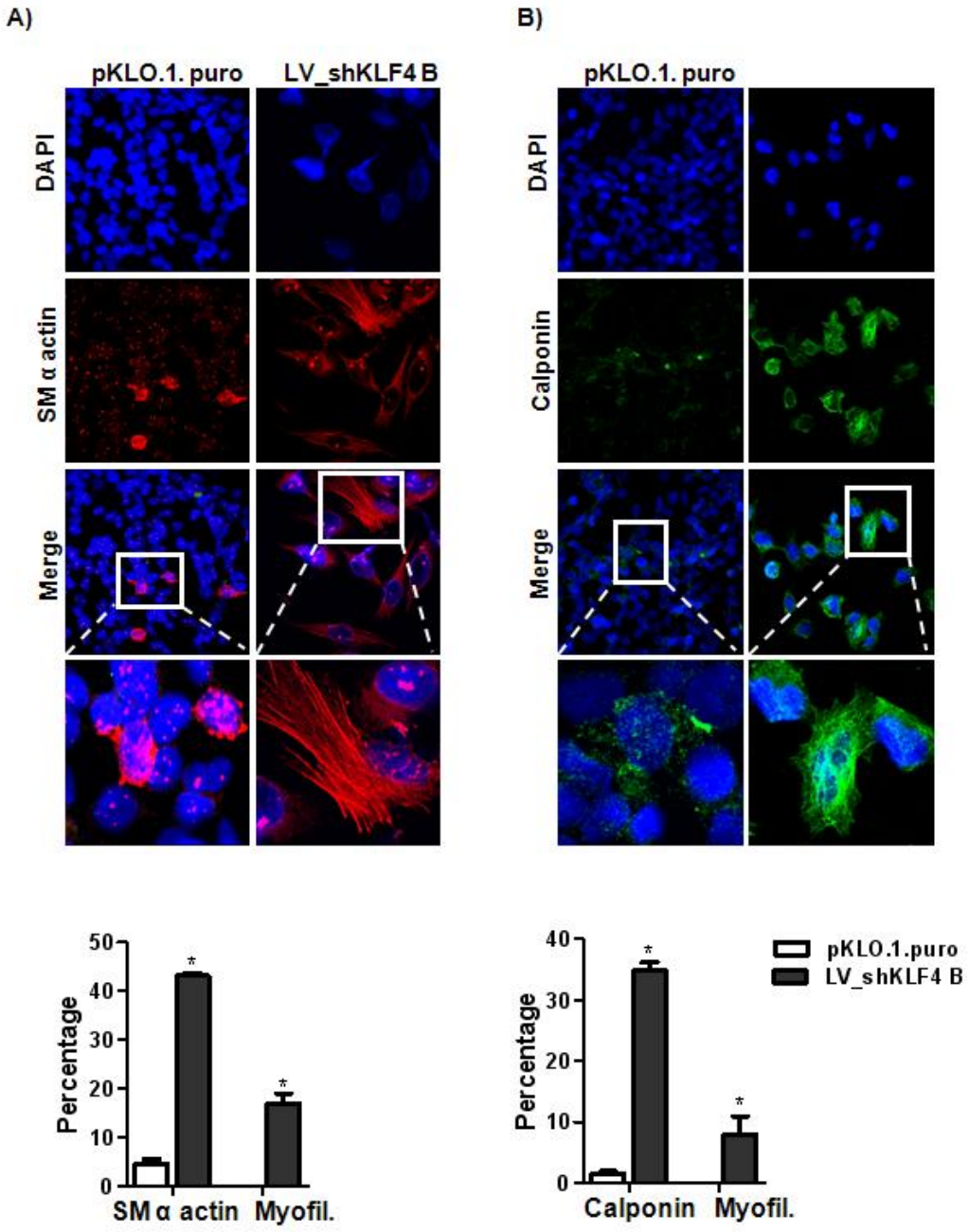


Figure 5-4. Differentiation of hSMSPC into smooth muscle cells through lentiviral mediated knock down of KLF4 upon TGF β1 stimulation. A) to determine whether knockdown of KLF4 induce SMC differentiation in hSMSPC cells, hSMSPC cells were infected with LV_shKLF4 B vector or an empty vector in differentiation media(SmGM2 1%FBS with TGF β1 5ng/ml). Puromycin was added after 24 hours post transduction and immunostained at day 7 for SM α actin (A) and calponin (B). The white boxed areas within the merged image are magnified in the lower panel.The confocal images showed the differentiation of hSMSPC into SMC in shKLF4 B transduced hSMSPC cells. Values are the means ± SD of three independent experiments. n=3, *P<0.05.

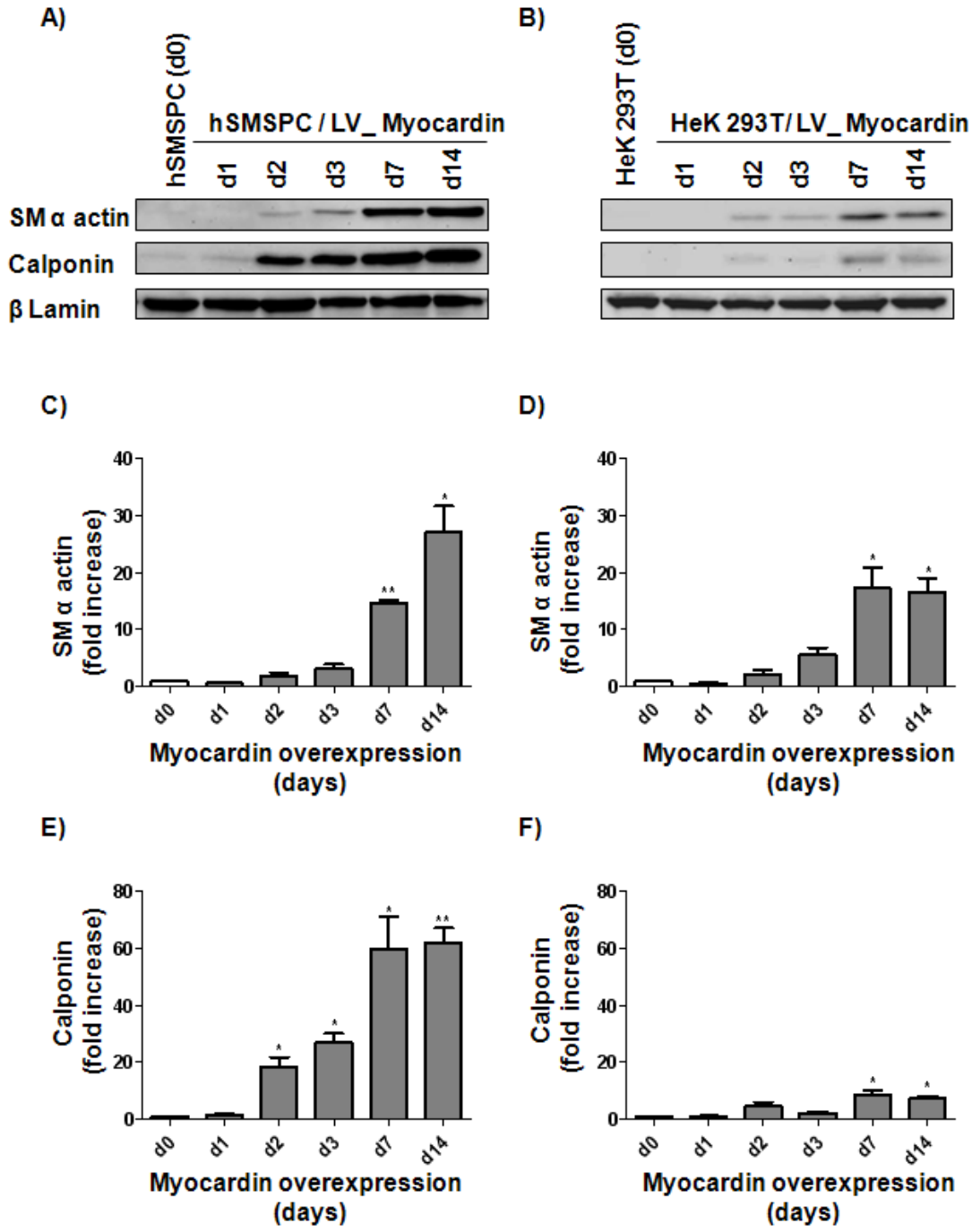
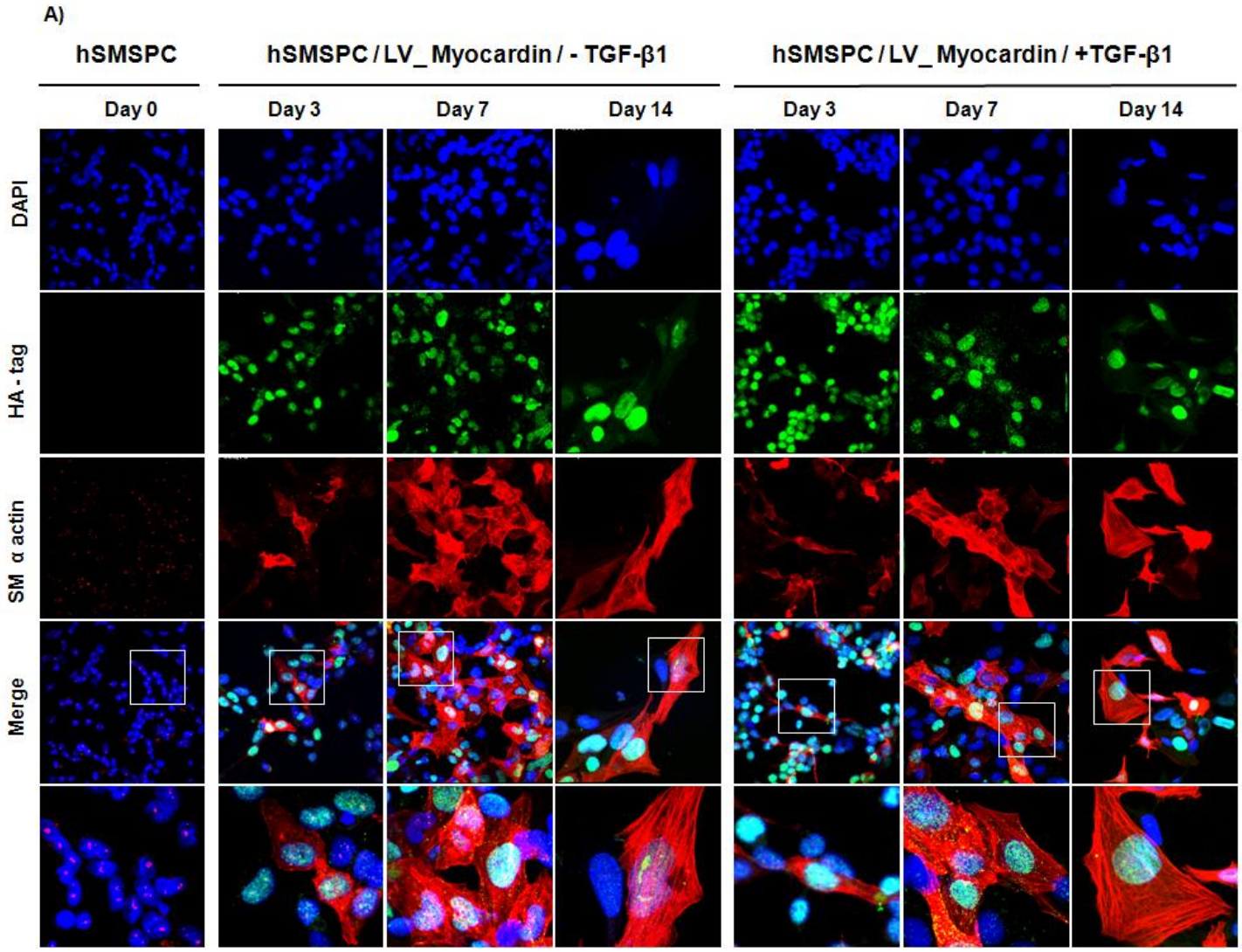


Figure 5-5. Overexpression of LV_myocardin induced smooth muscle marker proteins (SM α actin and calponin) over time, measured by western blot analysis in hSMSPC and HeK 293T cells. To determine whether forced expression of LV_myocardin can induce smooth muscle markers in hSMSPC and HeK 293T cells, the cells were infected with LV_myocardin and the transduced cells were harvested over time (do, d1, d2, d3, d7 and d14) and western blot analysis carried out which showed time dependent increase in the expression of SM α actin and calponin in both LV_myocardin transduced hSMSPC and HeK 293T cell (A&B). Graphical representation of densitometry quantification for the expression of SM α actin and calponin over time in LV_myocardin transduced hSMSPC cells and HeK 293T cells respectively (C, D, E and F). Protein levels were normalised to β lamin and to the control (day 0 untransduced cells). Data are representative of three independent experiments. n=3, *P<0.05**.**


A)
hSMSPC
hSMSPC / LV_ Myocardin / - TGF-β1
hSMSPC / LV_ Myocardin / +TGF-β1
Day 0
Day 3
Day 7
Day 14
Day 3
Day 7
Day 14
DAPI
HA - tag
SM α actin
Merge

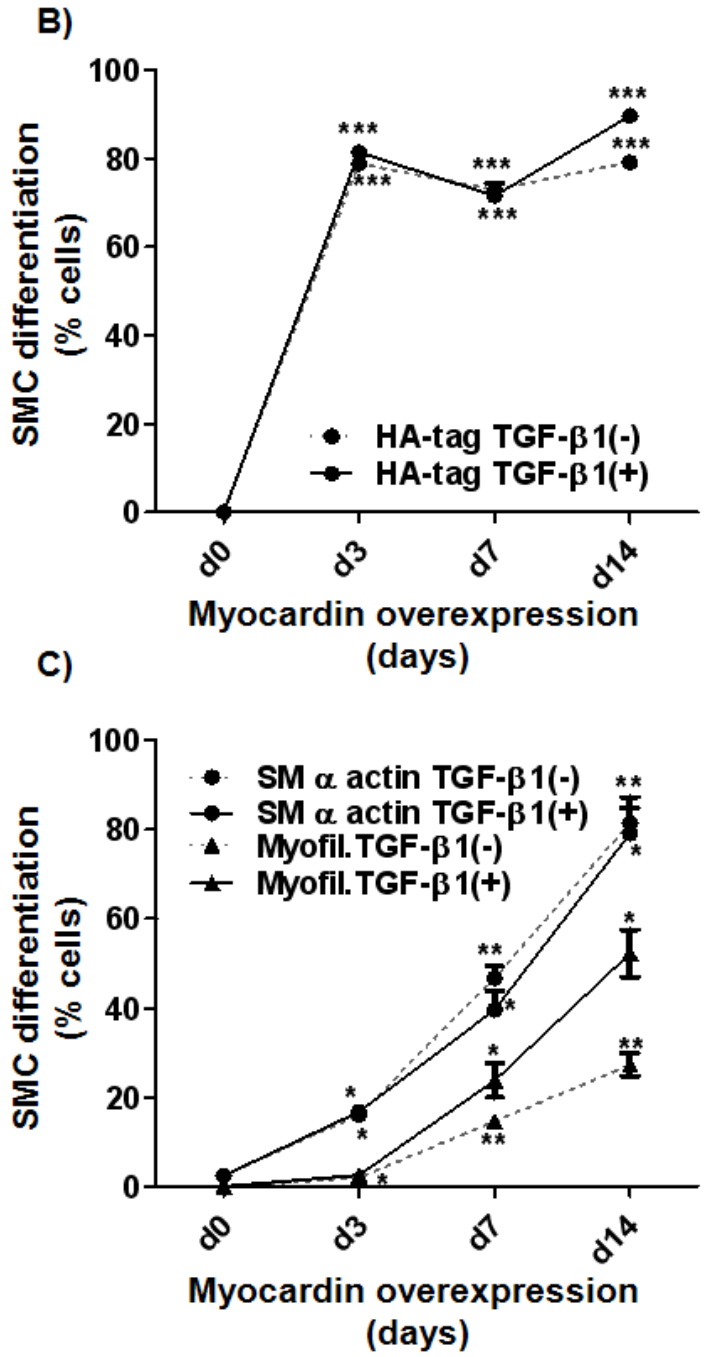
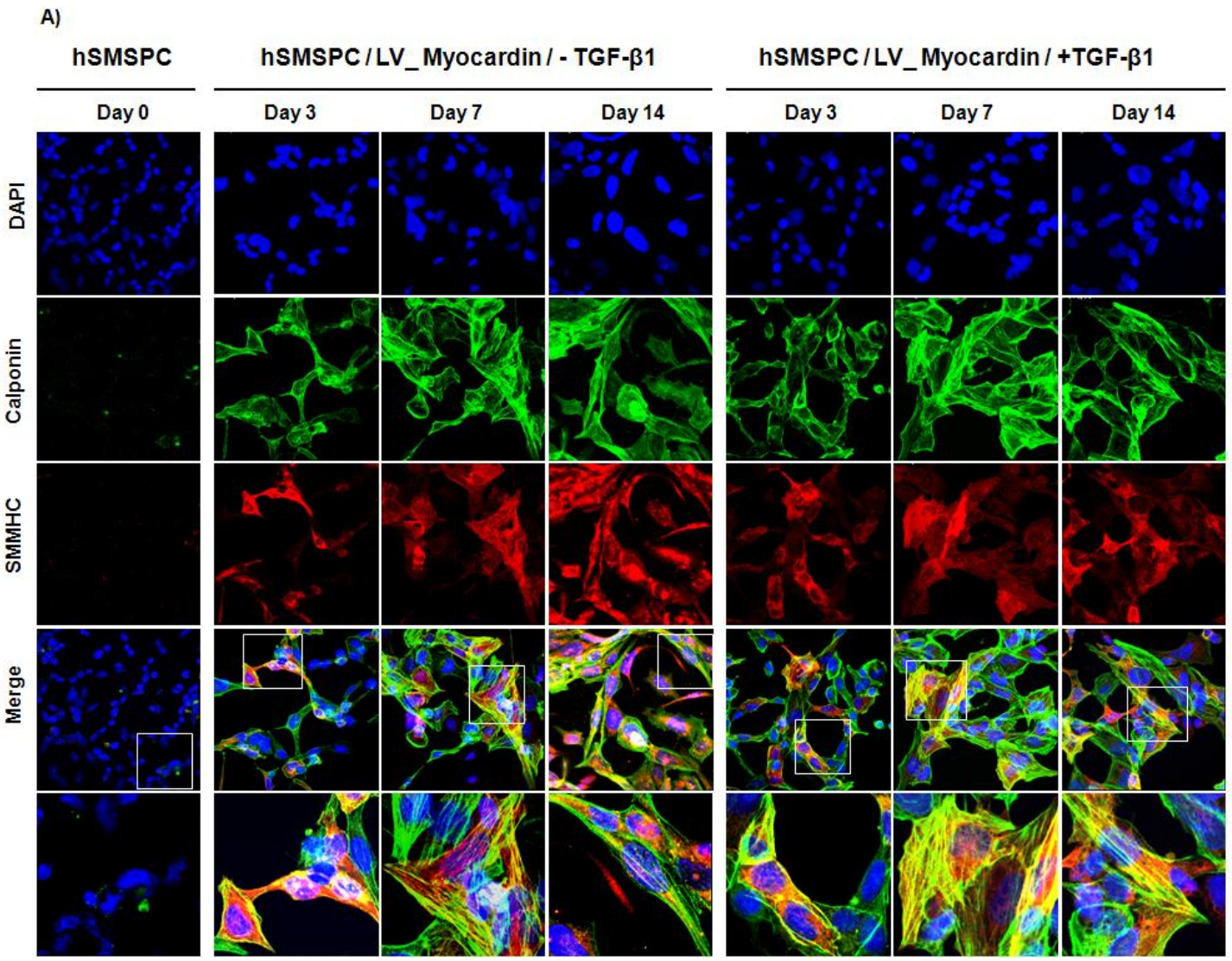


Figure 5-6. Forced expression of myocardin induced SMC differentiation with time dependent increase in SMA marker expression in hSMSPC cells. To examine whether forced expression of myocardin and TGF-β1 treatment can induce SM α actin expression in hSMSPC, the cells were transduced with 5 MOI of lentiviral particles encoding complete myocardin cDNA with or without TGF-β1 (5ng/ml). The transduced cells were fixed at various time points (3, 7, and 14 days post transduction) for immunofluorescence analysis with anti HA-tag and anti SM α actin antibody. A) Representative confocal images of *in vitro* SMC differentiation taken at different time points showing time dependent increase in SM α-actin expression in LV_myocardin transduced in both with or without TGF-β1 (5ng/ml) treatment in hSMSPC cells. The white boxed areas within the merged image are magnified in the lower panel B & C) Plotted are the percentage of HA-tag, SM α actin and myofilament relative to total DAPI positive cells in LV_myocardin with or without TGF-β1 (5ng/ml) treatment at indicated time points calculated from three separate experiments. n=3, ** P<0.01, *P<0.05.


A)
hSMSPC
hSMSPC / LV_ Myocardin / - TGF-β1
hSMSPC / LV_ Myocardin / +TGF-β1
Day 0
Day 3
Day 7
Day 14
Day 3
Day 7
Day 14
DAPI
Calponin
SMMHC
Merge

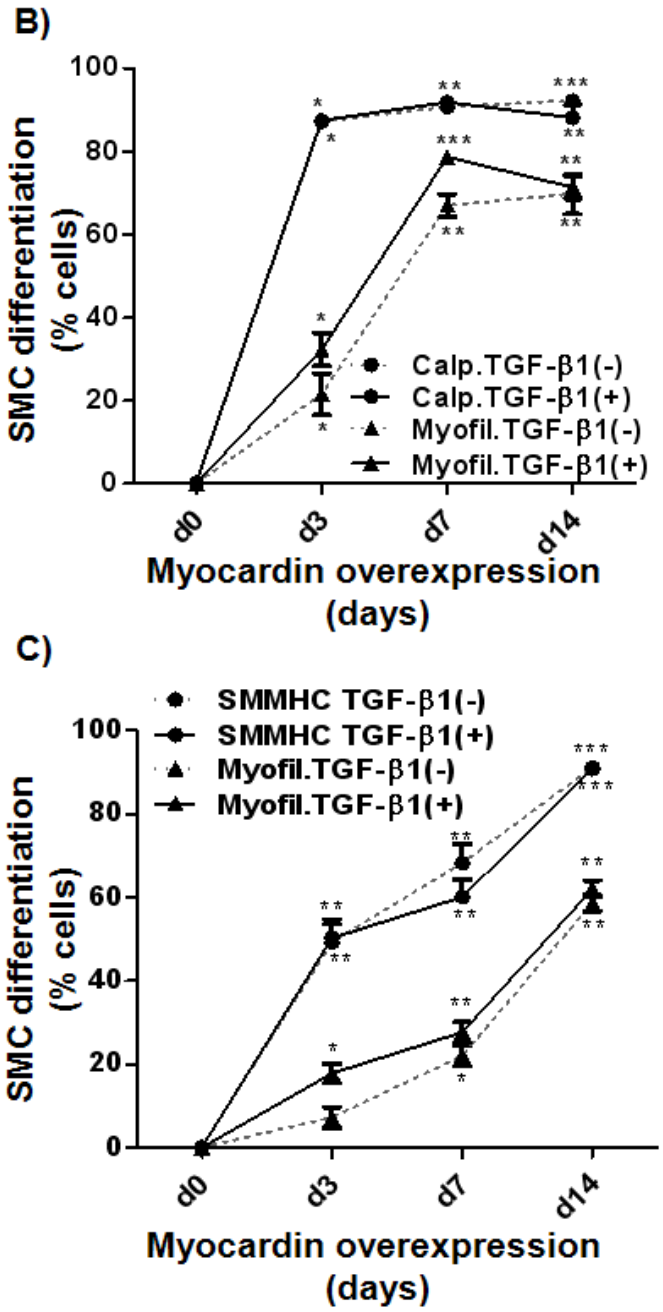
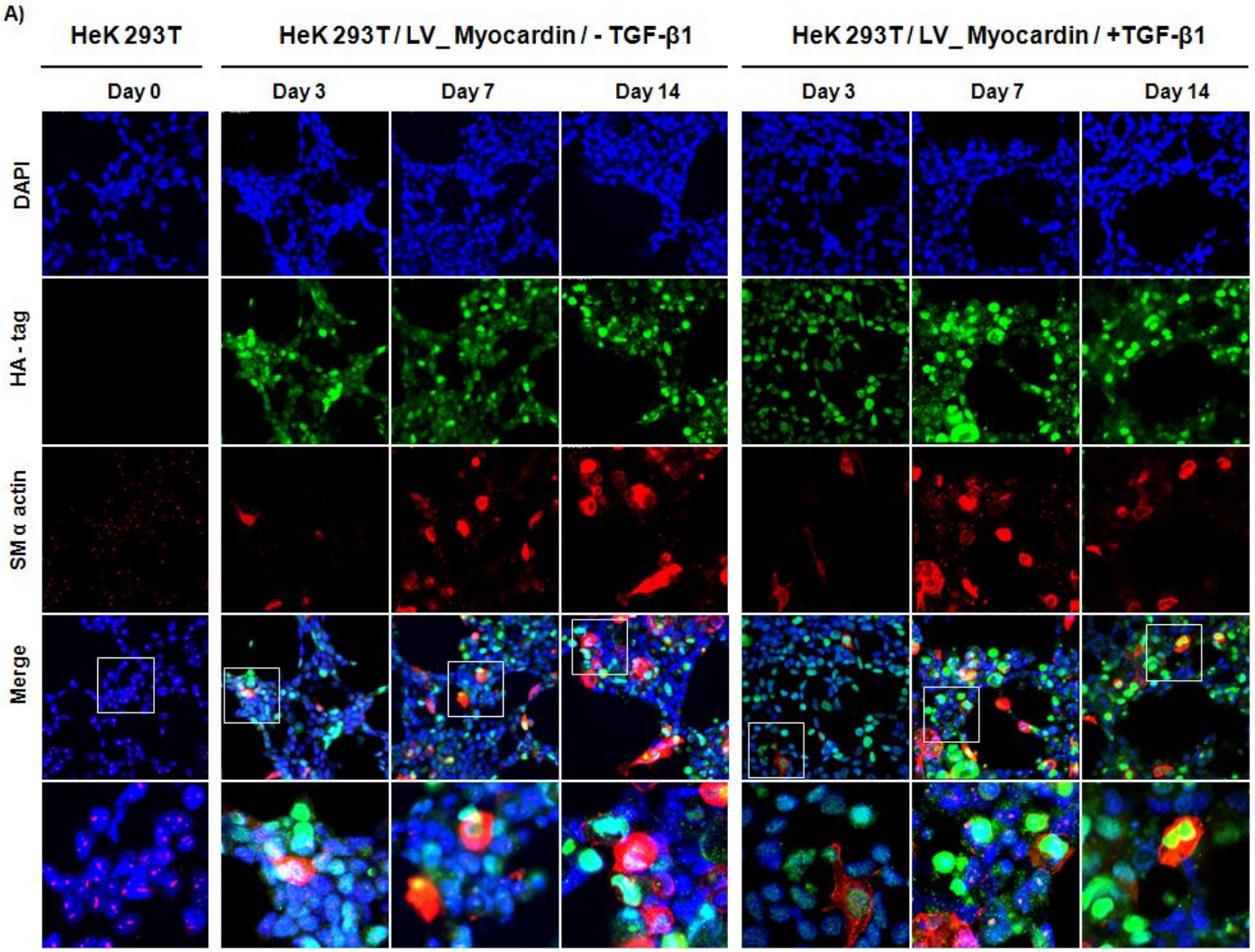


Figure 5-7. Forced expression of myocardin induced SMC differentiation with time dependent increase in calponin and SMMHC marker expression in hSMSPC cells. To examine whether forced expression of myocardin and TGF-β1 treatment can induce calponin and SMMHC expression in hSMSPC, the cells were transduced with 5 MOI of lentiviral particles encoding complete myocardin cDNA with or without TGF-β1 (5ng/ml). The transduced cells were fixed at various time points for immunofluorescence analysis with anti-calponin and anti SMMHC antibodies. A) Representative confocal images of *in vitro* SMC differentiation showing time dependent increase in calponin and SMMHC expression in LV_myocardin transduced in both with or without TGF-β1 (5ng/ml) treatment in hSMSPC cells. The boxed areas within the merged image are magnified in the lower panel B&C). Plotted are the percentage of calponin and SMMHC and their corresponding myofilament expression level relative to total DAPI positive cells in LV_myocardin with or without TGF-β1 (5ng/ml) treatment at indicated time points calculated from three separate experiments. n=3, ** P<0.01, *P<0.05.


A)
HeK 293T
HeK 293T / LV_ Myocardin / - TGF-β1
HeK 293T / LV_ Myocardin / +TGF-β1
Day 0
Day 3
Day 7
Day 14
Day 3
Day 7
Day 14
DAPI
HA - tag
SM α actin
Merge

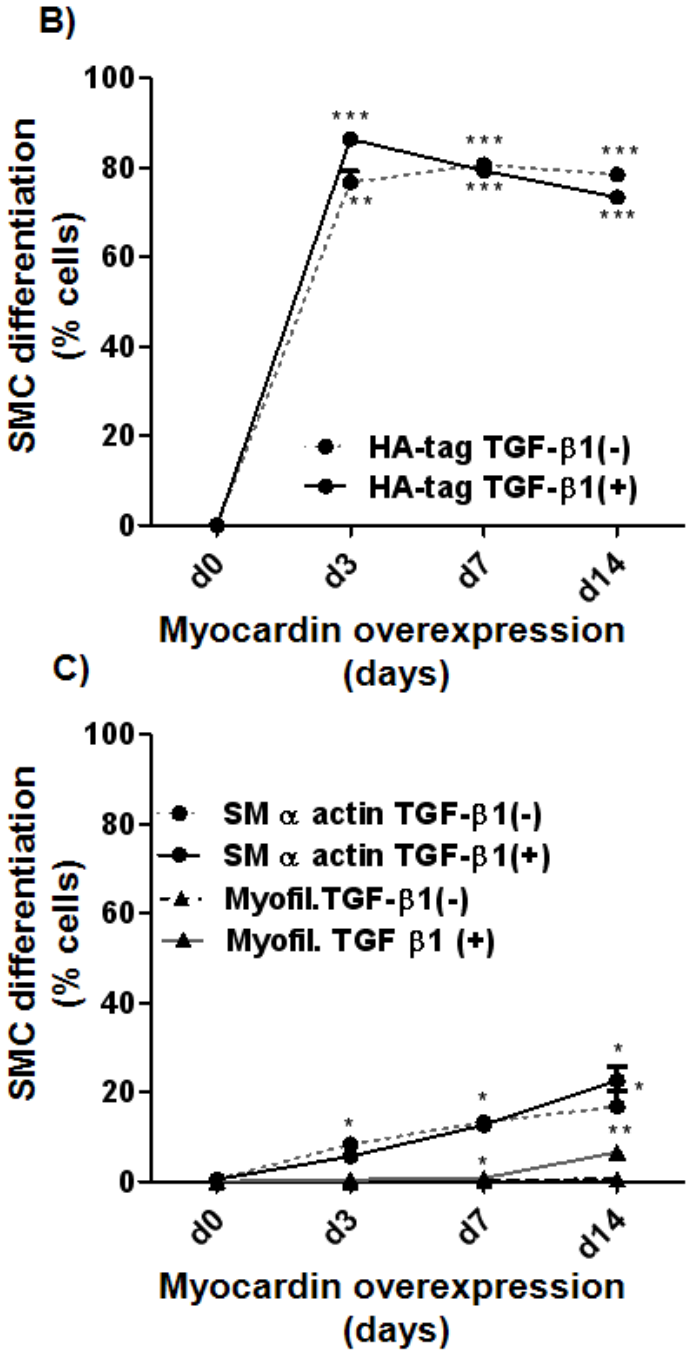
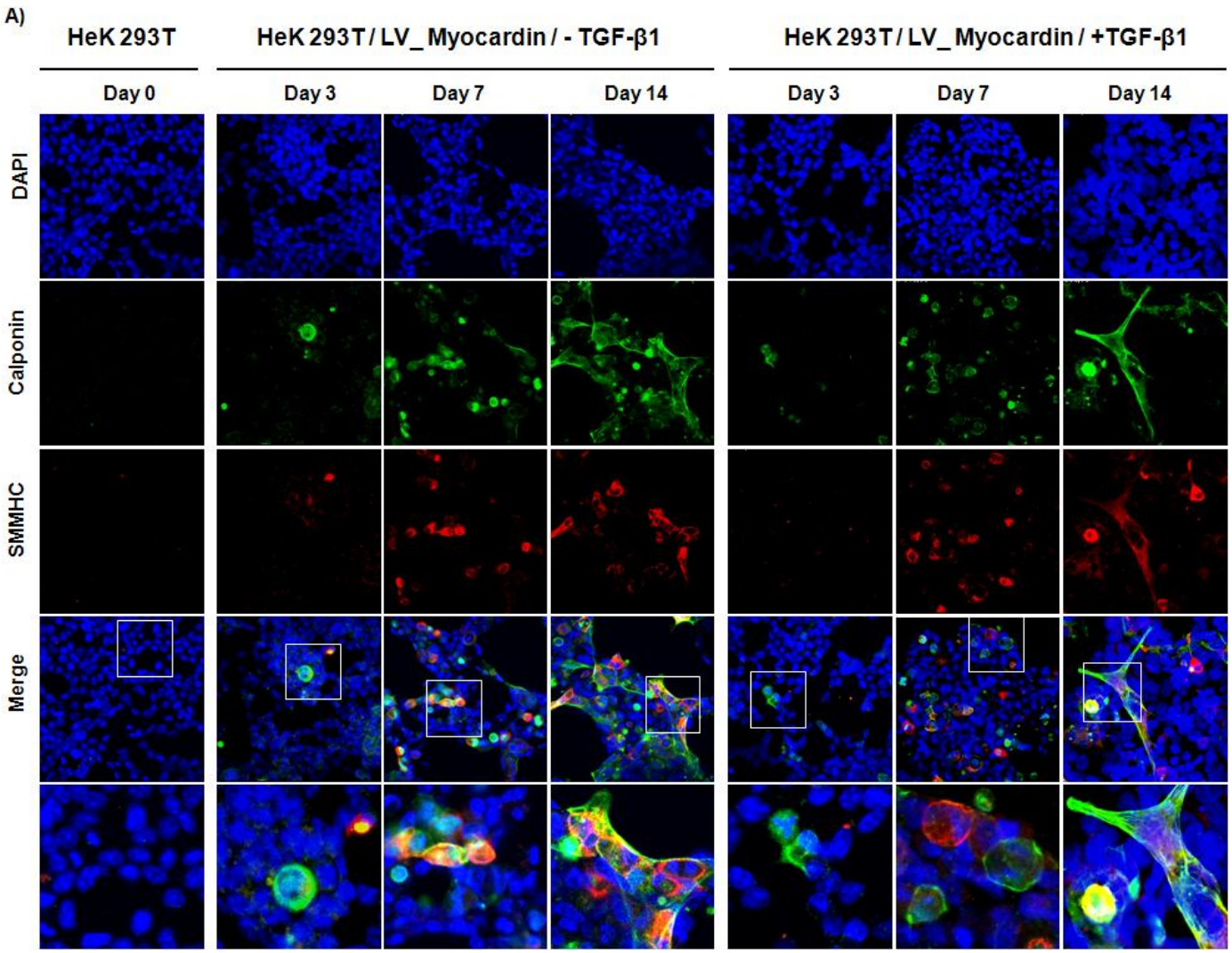


Figure 5-8. Forced expression of myocardin induced SMC differentiation to a lesser extent in HeK 293T cells. To examine whether forced expression of myocardin and TGF-β1 treatment can induce SM α actin expression in HeK 293T, the cells were transduced with 5 MOI of lentiviral particles encoding complete myocardin cDNA with or without TGF-β1 (5ng/ml). The transduced cells were fixed at various time points for immunofluorescence analysis with anti-HA-tag and anti SM α actin antibodies. A) Representative confocal images of *in vitro* SMC differentiation taken at different time points showing time dependent increase in SM α actin expression in LV_myocardin transduced in both with or without TGF-β1 (5ng/ml) treatment in HeK 293T cells. The white boxed areas within the merged image are magnified in the lower panel. B & C). Plotted are the percentage of HA-tag, SM α actin and myofilament relative to total DAPI positive cells in LV_myocardin with or without TGF-β1 (5ng/ml) treatment at indicated time points calculated from three separate experiments. n=3, ** P<0.01, *P<0.05.


A)
HeK 293T
HeK 293T / LV_ Myocardin / - TGF-β1
HeK 293T / LV_ Myocardin / +TGF-β1
Day 0
Day 3
Day 7
Day 14
Day 3
Day 7
Day 14
DAPI
Calponin
SMMHC
Merge

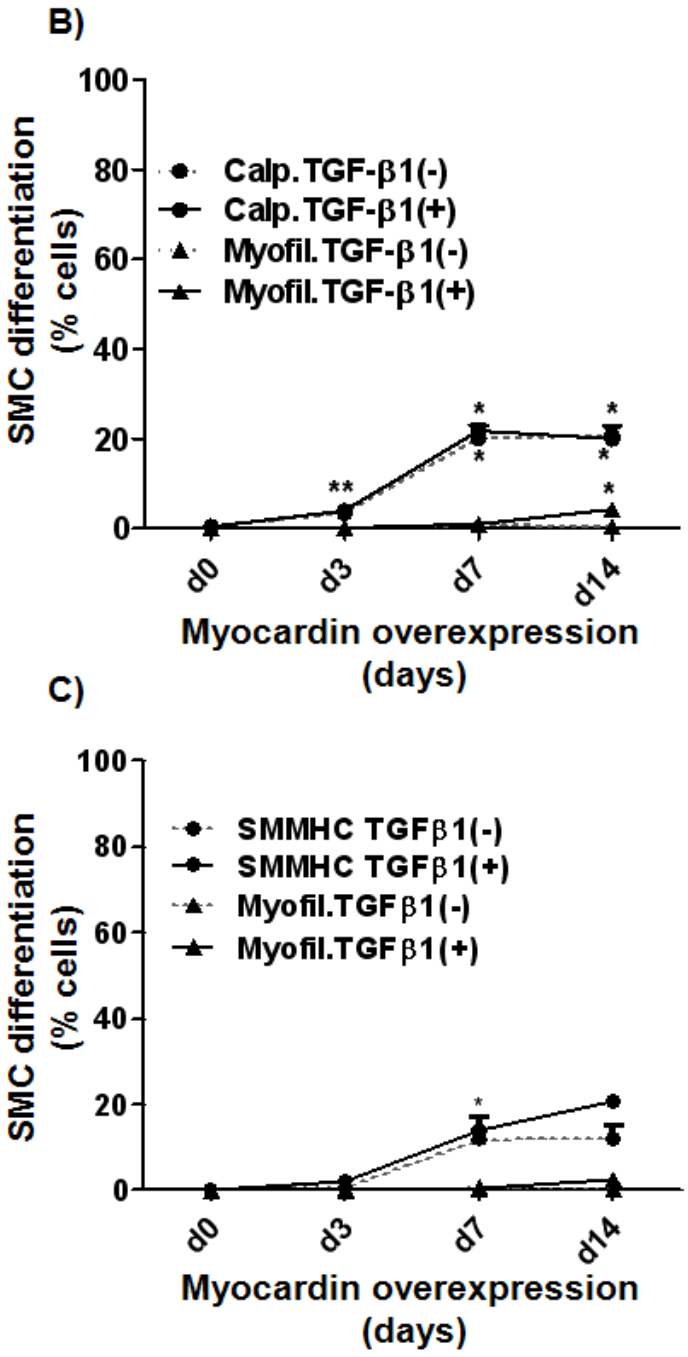


Figure 5-9. Forced expression of myocardin induced SMC differentiation to a lesser extent in HeK 293T cells. To examine whether forced expression of myocardin and TGF-β1 treatment can induce calponin and SMMHC expression in HeK 293T, the cells were transduced with myocardin lentiviral particles with or without TGF-β1 (5ng/ml). The transduced cells were fixed at various time points for immunofluorescence analysis with anti-calponin and anti SMMHC antibodies. A) Representative confocal images of *in vitro* SMC differentiation showing time dependent increase in SM α actin expression in LV_myocardin transduced with or without TGF-β1 treatment in HeK 293T cells. The white boxed areas within the merged image are magnified in the lower panel. B & C are plotted percentage of calponin and SMMHC and myofilament relative to total DAPI positive cells in LV_myocardin with or without TGF-β1 treatment at indicated time points calculated from three separate experiments. n=3, ** P<0.01, *P<0.05.

5.4. Discussion

The purpose of the current study was to investigate the effect of TGF-β1 mediated SMC differentiation in KLF4 knockdown and myocardin overexpressed hSMSPC cells. We hypothesized that the presence of KLF4 in hSMSPC results in blockade of SMC differentiation through repressing myocardin. Thus, either knockdown of endogenous KLF4 or overexpression of myocardin can induce SMC differentiation in hSMSPC cells. To test this hypothesis, initially the expression level of KLF4 was compared to that in SMC by both immunostaining and western blot analysis. Then, the expression of KLF4 was downregulated using shRNA targeting KLF4 or myocardin was overexpressed using lentiviral vector and stimulated with differentiation medium for seven days. The results revealed that hSMSPC has higher levels of KLF4 as compared to SMC and that silencing of KLF4 increased the SMC differentiation of hSMSPC in response to TGF-β1 stimulation as measured by immunostaining of smooth muscle specific markers SM α-actin and calponin. Furthermore, overexpression of myocardin induced complete SMC differentiation of hSMSPCs with upregulation of myofilamentous smooth muscle markers such as SM α actin, calponin and SMMHC, effects moderately augmented by TGF-β1 stimulation at late stage time points.

Epigenetic modification of histones H3 & H4 lysine's within the CArG boxes controls the cell specific activation of SMC genes [98]. Histone modification of CArG element enables the accessibility to SRF which in turn recruits its cofactor myocardin and transactivates smooth muscle specific SMC gene expression [98]. KLF4 is a known transcriptional repressor for SMC genes and has been shown to reduce SRF binding ability to CArG element within the SM α actin promoter [167]. KLF4 also represses myocardin-induced smooth muscle differentiation marker gene expression in SMC [138, 140, 360]. KLF4 binds to TCE element adjacent to CArG elements, directly recruit histone deacetylase to SMC promoters and renders chromatin inaccessible to SRF [138]. Furthermore, the results outlined in previous work showed that KLF4 can directly bind to the myocardin promoter and repress transcription of myocardin gene expression in hSMSPC cells. Collectively, these data suggest that presence of abundant KLF4 in hSMSPC might block SRF access to the SMC promoters causing a reduction myocardin-induced SMC differentiation in hSMSPC cells.

The presence of two CArG elements, in combination with a TCE element, has been shown to be necessary for TGF-β1 inducibility of smooth muscle marker gene transcription [237]. In addition, TGF-β1 was also shown to increase SRF

expression and binding to CArG elements within SMC gene specific promoters [361]. Interestingly in our own studies, knockdown of KLF4 in differentiation media induced myocardin expression in hSMSPCs (Chien ling Huang, 2013, personal communication) suggesting that silencing of KLF4 followed by TGF-β1 stimulation induced SRF /myocardin SM gene transcription in hSMSPCs. It is conceivable that reduction in KLF4 binding to TCE elements might have allowed SRF access to CArG elements and led to the further activation of myocardin/SRF/CArG-dependent SMC genes in hSMSPCs

Several studies have demonstrated that siRNA mediated knockdown of KLF4 resulted in ablation of both oxidised phospholipids and PDGF-BB induced downregulation of smooth muscle differentiation marker transcription in SMCs [167, 362]. In transgenic mice, conditional KLF4 knockout delayed the repression of SM marker genes following vascular injury [360]. Recent studies have demonstrated that knockdown of KLF4 can also induce differentiation of ES cell differentiation [338]. Similarly, in this study, KLF4 downregulation in hSMSPC through shRNA lentiviral vector increased SMC differentiation. Moreover, silencing of KLF4 alone promoted hSMSPC differentiation into SMCs, suggesting that hSMSPC contain the basic transcription machinery and cofactors required for SMC differentiation but are kept in an undifferentiated state by the transcriptional repressor KLF4.

KLF4 acts as a master regulator for pluripotency core transcription factors including Oct 4, Sox 2 and Nanog [338]. In addition, overexpression of KLF4 maintains the undifferentiated state of embryonic stem cells. Of interest, human myocardin promoter also has putative binding sites for other stem cell transcription factors such as Oct 4, Sox 2 and Nanog within the -4007 bp upstream to 5' of the TSS. Thus, knockdown of KLF4 along with other stem cell factors that may bind the human myocardin promoter could further enhance the commitment of hSMSPC to differentiated SMCs.

A basic structural hallmark of contractile SMC is the presence of myofilament and growth cessation [27]. Myofilament bundles are essential for the contractile phenotype of differentiated SMC and represent an important step along the SMC phenotype switching pathway. Myocardin, a SAP domain transcription factor, acts as a SRF cofactor and is expressed in cardiomyocytes and in vascular and visceral SMCs from embryonic to postnatal development [145]. Overexpression of myocardin induces growth arrest and enhances spindle shape morphology unique to SMCs with enriched myofilament expression in BC3H1 cells [363]. Similarly, the SMC progenitor cell line 10T1/2 also displays low levels of myocardin expression

and undergoes differentiation to a contractile SMC phenotype by forced expression of myocardin [94]. Furthermore, myocardin is activated by TGF-β1 in 10T1/2 cells [240]. Since, myocardin and TGF-β1 have both been shown previously to induce SMC differentiation, the combined effect of myocardin and TGF-β1 was examined on SMC differentiation of hSMSPC cells. Interestingly, overexpression of myocardin showed growth arrest and increased spindle morphology in hSMSPCs, but not in HeK 293 T cells, suggesting that smooth muscle specific molecular cofactors and transcriptional machinery needs to be present for phenotype modulation to occur.

While the expression profiles of baseline smooth muscle markers were similar between undifferentiated hSMSPC and HeK 293T cells, following LV_myocardin transduction, the expression of smooth muscle lineage markers was significantly increase in hSMSPC in comparison to in HeK 293T cells. More specifically, expression of the widely recognised SMC differentiation specific markers calponin and SMMHC were efficiently induced by forced myocardin expression in hSMSPCs indicating that the molecular machinery responsible for the SMC differentiation program are likely to be present in hSMSPCs and not in HeK 293 T cells. Furthermore, the relative percentage levels of Myocardin-HA-tagged cells in hSMSPCs and HeK 293 T cells (Fig. 7.2 & Fig. 7.4) was confirmed showing that both cell types to be equally expressing myocardin reaffirming that although expressing myocardin at high levels, without the necessary molecular machinery for SMC differentiation as must be present in hSMSPCs, phenotypic modulation does not occur efficiently.

Calponin was the first SMC marker detected at day 1 following LV_myocardin transduction, followed by increases in SM α actin and SMMHC at day 3 in hSMSPC cells. In contrast, SM α actin was the first SMC marker detected at day 3 post-LV_myocardin transduction whereas calponin and SMMHC were only evident at day 7 in HeK 293T cells, suggesting that the low levels of myocardin gene expression contributes to the undifferentiated state of hSMSPC cells. The findings of the current study were consistent with those of Chen *et al*., [143] who found that myocardin activates the highly specific smooth muscle markers, SM-calponin and SMMHC to a greater extent in SMC than in non SMC. In contrast, myocardin transactivation of the less specific markers SM α actin and SM22α is higher in non SMC compared to SMC [143] further demonstrating that enhanced calponin and SMMHC expression is the bench mark for SMC differentiation.

The presence of an unique epigenetic modification (H3K4dMe) has been detected at SMC gene promoters in SMCs but not in non SMCs [138]. While

myocardin does not bind directly to CArG elements to induce SMC transcription, it does stabilize SRF binding to CArG elements in an H3K4dMe-dependent manner [98, 138]. Therefore, indicating that the presence of SMC promoter specific histone methylation along with smooth muscle restricted expression of myocardin enables transactivation of myocardin/SRF/CArG-dependent SM specific gene transcription in SMC but not in non-SMCs. Herein it was demonstrated that overexpression of myocardin induced SMC differentiation in a SMC specific manner. Thus it would be interesting to determine if the myocardin-mediated SMC differentiation of hSMSPCs was H3K4dMe-dependent providing a compelling epigenetic basis for the SMC differentiation capacity of hSMSPCs or indeed other SMC progenitor cells.

The smooth muscle specific phenotypic changes following lentiviral mediated overexpression of myocardin was routinely quantified at protein levels using Western blot as is standard throughout the literature. However, superseding these findings, we also measured myofilament levels using immunofluorescence in hSMSPC and HeK 293T cells. Myofilament is the structural and functional hallmark specific to contractile SMC phenotype and therefore a better indicator of SMC-like phenotype. Interestingly, overexpression of myocardin resulted in a significant increase in the stellate cells with spindle morphology in hSMSPC cells and induced SMC differentiation to a far greater extent than in HeK 293T cells, with expression of myofilamentous SM markers including SM α actin, calponin and SMMHC indistinguishable from that of SMCs. These findings demonstrate that hSMSPCs can be differentiated into SMC-like cells. However, to determine if fully differentiated hSMSPCs have similar functionality to that of adult SMCs, contraction assays and calcium signalling as described by Simper *et al*.,[245] would need to be performed.

In summary, these results suggest that KLF4 acts as transcriptional repressor blocking smooth muscle differentiation of hSMSPCs and that silencing of KLF4 augments TGF-β1-induced SMC differentiation of hSMSPCs. Furthermore, lentiviral-mediated forced expression of myocardin alone or in combination with TGF-β1 induces smooth muscle lineage specific differentiation of hSMSPCs to SMC-like cells that display myofilament levels indistinguishable from adult SMCs.

6. Specific Aim 4 - Determination of tissue capsule forming potential of rSMSPC eGFP cells using a rat model

6.1. Introduction

Atherosclerosis is a major cause of coronary artery disease leading to myocardial ischemia, stroke and aortic aneurysm. Atherosclerotic lesions develop due to a chronic inflammatory reaction to excessive lipid deposition in the arterial wall which leads to arterial damage [364]. A major therapy for diseased coronary artery involves replacement of damaged arteries with small diameter vascular grafts. The saphenous vein, radial artery and mammary artery from the patients are commonly used autografts for vessel replacement [314]. Although, autografts may be non thrombogenic and highly flexible, repeat bypass procedures, size or length availability are always limitations associated with their use. Synthetic grafts such as those made from dacron fabric grafts and expanded polytetrafluoroethylene (ePTFE) have been invented to overcome the limited availability of autografts [365]. However, synthetic grafts initiate a foreign body reaction in small diameter vessels, cause neointimal hyperplasia and their usage is limited [366, 367].

Implantation of sterile materials such as boiled liver, egg white, filter membrane or boiled blood clot into the peritoneal cavity of animals like dogs, rats or rabbit results in their encapsulation with multiple layers of granulated tissue [368-370]**.** Insertion of a foreign object initiates an inflammatory reaction which involves cytokines, chemokines and matrix metalloproteinases. These soluble mediators further mediate a cascade of cellular processes such as cellular activation, angiogenesis, migration and recruitment of inflammatory cells to the site [371] and over a period of time, these reaction results in formation of multi-layered tissue encapsulation of foreign bodies which is mainly composed of myofibroblasts and lined by endothelial like cells [367, 372]. Use of tissue granulation in response to implantation of sterile silastic tubes (10mm length with 3mm diameter) in the peritoneal cavity of animal [367] provides a new scope for tissue engineering of vascular grafts.

Insertion of free floating silastic tubes into the peritoneal cavity of rat, rabbit or dog induces the tissue granulation covering the entire tube within 2 weeks. The tissue capsule resembles hollow tubes with inner mesothelial lining and a media with multiple layers of myofibroblast like cells [367, 373]**.** These tissue capsules harvested from the animals own peritoneal cavity has been successfully grafted in the carotid, femoral or abdominal artery [367, 373]**.** Since, tissue granulation is autologous problems associated with tissue rejection are overcome [374]. In due course, the cells located in the grafted tissue capsule respond to the local environmental cues and remodel into structures similar to the native blood vessel

[374]. In 3-4 months, grafted tissue capsule increases in thickness with formation of adventitia and lamellae and the myofibroblasts present in the media of tissue capsule further differentiate into smooth muscle cells similar to the nearby artery [367] . These results suggest that tissue capsules harvested from peritoneal cavity can be used as artificial arteries in arterial reconstruction surgery for replacing the damaged arteries.

Previous research findings demonstrated that knockdown of KLF4 and overexpression of myocardin induced SMC differentiation of hSMSPC cells *in vitro*. Therefore, this study **hypothesis that rSMSPC expressing eGFP can integrate into tissue capsule structures *in vivo* and contribute to their cell wall content**.

The present study aims to establish a rat model to study the possibility of rSMSPC eGFP cell integration within the peritoneal derived tissue capsule.

6.2. Methods

6.2.1. Silastic tube implantation and harvest of tube capsule

Fisher hPAP rats were anesthetized using urethane (1.25 g/kg of body weight) and a small (2 inch) abdominal incision was made in cleanly shaved rat abdomen. Irradiated 10 mm lengths of 3 mm diameter silastic tubes were placed inside a 50 ml sterile falcon and coated with fibronectin at 5 µg/cm^2 concentration for 45 minutes at room temperature on a rocker. Then, four 10 mm length uncoated silastic tubes or coated with fibronectin or fibronectin plus 50 million rSMSPC eGFP cells were implanted inside the peritoneal cavity of each animal. Sterile saline (3 ml) was injected to allow the tubes to float freely inside the peritoneal cavity. Two weeks after implantation, the rats were anesthetized using urethane and examined for tube capsule formation. The tissue capsules were removed from the tubes and processed for eGFP detection, H&E staining and immunohistochemical analysis of smooth muscle marker protein expression.

6.2.2. Tissue digestion and FACS analysis

Total cell suspension of harvested tissue capsules were obtained by collagenase enzymatic digestion. The tissue capsules were dissociated by cutting into small pieces and dispersed in RPMI-1640 medium supplemented with freshly made collagenase (2mg/ml). The enzymatic digestion was initiated by incubating the lysate for 30 minutes at 37^{O}C on an orbital shaker with 1200 rpm. The enzymatic reaction was stopped by addition of RPMI-1640 with serum and cells were pelleted

by centrifugation at 600g for 5 minutes at 4^{O}C. The cells were suspended in FACS buffer and filtered through cell strainer for eGFP FACS analysis.

6.2.3. Hematoxylin and Eosin (H&E) staining protocol

The tissue capsule was tied at one end with silk suture and optimal cutting temperature (OCT) medium was slowly injected through the other end filling the whole tube (Fig.8.1C). The pre-frozen tissue capsules were placed into moulds with OCT, liquid embedding material and frozen into hardened blocks for sectioning. The embedded frozen capsules were sliced to 5 µm thickness sections using cryostat for histological analysis. The OCT sections were allowed to thaw briefly at room temperature and fixed with 4% paraformaldehyde for 10 minutes at 4^{o}C. The slides were washed once with PBS for 5 minutes. The slides were immersed in Mayer's hematoxylin Z solution for 5 minutes and dipped in 1% acid alcohol for 10 seconds then washed in running tap water for 5 minutes. The sections were stained with eosin for one minute and then sequentially dehydrated with 90% and 100% alcohol concentrations for five minutes. The sections were cleared in xylene for a minute, dried and mounted under a cover slip with DPX mounting media.

6.3. Results

To demonstrate that SMSPC can integrate into neovascular structures *in vivo* and contribute to their myofibroblasts like content, silicon tubes were implanted as uncoated or coated with fibronectin or fibronectin coating plus 50 million rSMSPC eGFP cells (mixed in 3ml saline) into rats peritoneal cavity and tissue capsules were harvested 14 days post implantation (Fig. 6.1 A(i-iii)).

The uncoated silastic tubes showed an inflammatory response with 32% tissue encapsulation. However, fibronectin coated silastic tubes injected with or without 50 million rSMSPC eGFP cells induced tissue granulation over the entire length of tube with 66% and 73% (Fig. 6.1B) tissue encapsulation, respectively. The tissue capsules harvested from fibronectin coated with 50 million rSMSPC eGFP cells showed a thick tissue granulation when compared to fibronectin only coated tubes. However, there was no significant difference in tissue capsule formation between fibronectin coated silastic tubes injected with or without 50 million rSMSPC eGFP cells (Fig. 6.1B). The tissue capsules were separated carefully from the tubes and used for subsequent analysis. Of interest, when the whole tissue capsule was checked for eGFP cells, patches of eGFP cells were detected on the tissue capsule from fibronectin coated tubes injected with 50 million rSMSPC eGFP cells using confocal microscopy (Fig.6.1 E).

To examine the integration of rSMSPC eGFP cells within the tissue capsule, the capsule harvested from fibronectin coated tubes injected with or without 50 million rSMSPC eGFP rats were subjected to tissue digestion with collagenase enzyme. The tissue capsule harvested from fibronectin coated tubes was used as control.

A small number of eGFP positive cells (~1%) were detected by FACS analysis in fibronectin coated tube capsules injected with 50 million rSMSPC eGFP cells (Fig. 6.1E). Subsequent confocal microscopic analysis of frozen sections from uncoated, fibronectin coated tubes injected with or without rSMSPC eGFP cells showed localisation of few eGFP cells within tissue capsules from fibronectin coated tubes injected with rSMSPC eGFP (Fig. 6.2), which is consistent with the results from the tissue digestion.

In order to study the structure of tissue capsules developed in the peritoneal cavity, frozen sections of 5 µm thickness from uncoated or fibronectin coated tubes injected with or without 50 million rSMSPC eGFP were stained with hematoxylin and eosin stain (Fig. 6.3). The tissue capsule section from uncoated tubes (Fig. 6.3A), showed a very thin layer of tissue encapsulation with scattered cells throughout the capsule. However, tissue capsule section from fibronectin coated tubes with or without 50 million rSMSPC eGFP cells injected rats showed a homogenous thick layer of tightly packed cells covering the length of the tube (Fig. 6.3B&C).

To investigate the SMC content of the harvested tissue capsules, frozen sections from uncoated or fibronectin coated tubes injected with or without 50 million rSMSPC eGFP were assessed by immunofluorescence staining for anti- SM α actin and anti-calponin. As shown in Fig. 6.4 A (i), tissue capsule from uncoated tube stained positively for SM α actin but negatively for calponin (Fig. 6.4B (i)), The capsules from both fibronectin coated tubes with or without 50 million rSMSPC eGFP cells from the injected rats were stained positive for both SM- α actin and calponin (Fig. 6.4 A (i&ii) and Fig. 6.4 B (i&ii). However, expression of calponin was higher in capsules from fibronectin coated with 50 million cells injected rats compared to fibronectin coated tube capsules.

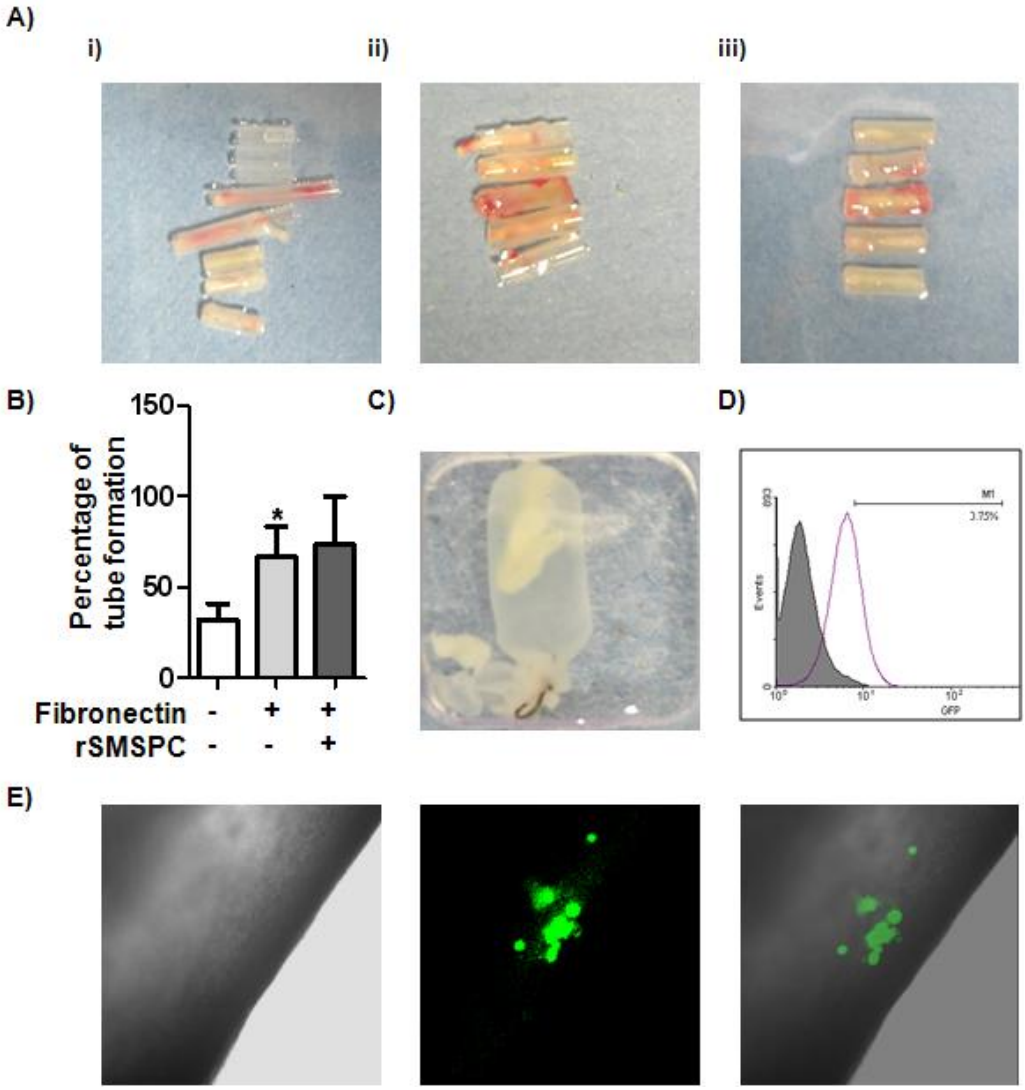


Figure 6-1. Tubular tissue capsule formation in rat's peritoneal cavity. To investigate the role of rSMSPC eGFP cells integration during neovessel formation *in vivo* in rats peritoneal cavity, silastic tubes uncoated or fibronectin coated tubes injected with or without 50 x 10^6 rSMSPC eGFP cells was implanted into rats peritoneal cavity. A) Tissue capsule harvested from A-i) uncoated silastic tubes ii) fibronectin coated silastic tubes and iii) fibronectin coated silastic tubes with 50 x 10^6 rSMSPC eGFP cells after 2 weeks of implantation. B) Percentage of tubes formation in different treatments. C) Pre frozen tissue capsule placed into a mold with OCT, liquid embedding material. D) Percentage of rSMSPC eGFP cells detected by FACS analysis in tissue capsule harvested from fibronectin coated silastic tubes injected with 50 x 10^6 rSMSPC eGFP cells. E) Capsule from fibronectin coated tubes injected with 50 x 10^6 rSMSPC eGFP cells showing a patch of rSMSPC eGFP cells integrated within tissue capsule harvested after 2 weeks of implantation. (Right – whole tissue capsule; middle panel-patches of eGFP cells and left panel- merge). Data are representative of three independent experiments. n=3, *P<0.05.

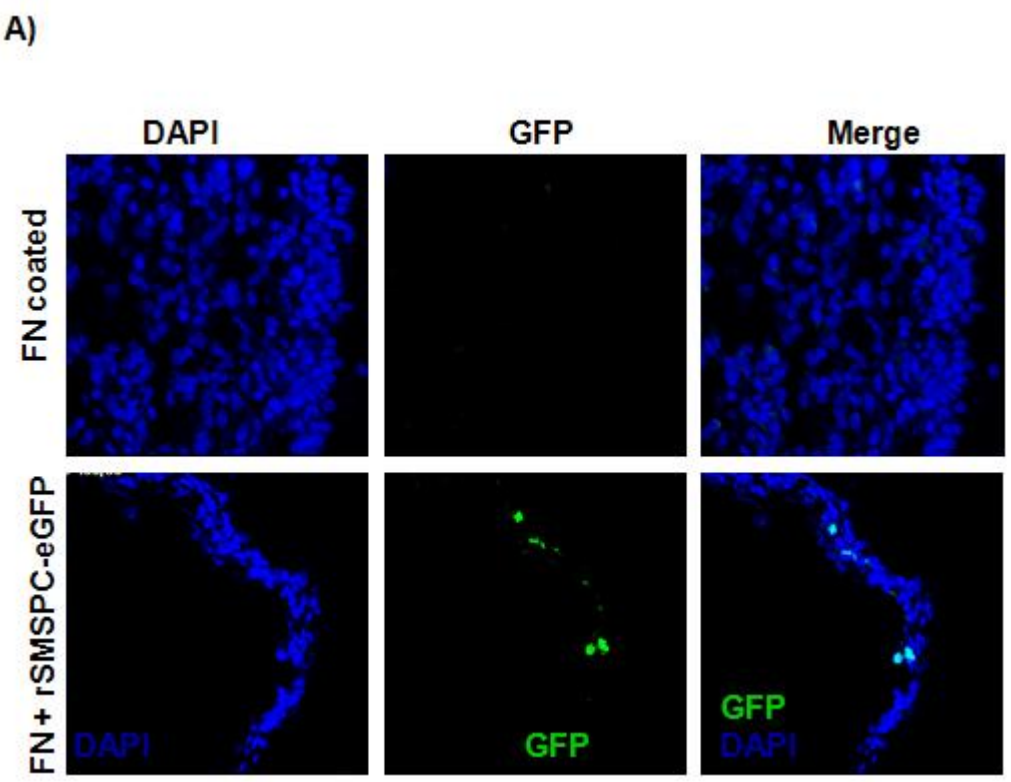


Figure 6-2. Detection of rSMSPC GFP integration within the tissue capsule.

To determine the number of rSMSPC eGFP cells integration directly in tissue capsule formation, fibronectin coated (top panel) or fibronectin coated with rSMSPC eGFP $50x10^6$ cells injected (bottom panel) frozen sections were analysed for the presence of eGFP cells by confocal microscopy. A small number of rSMSPC eGFP cells were found localised within the tissue capsule obtained from rSMSPC eGFP injected rats. Data are representative of three independent experiments. n=3.

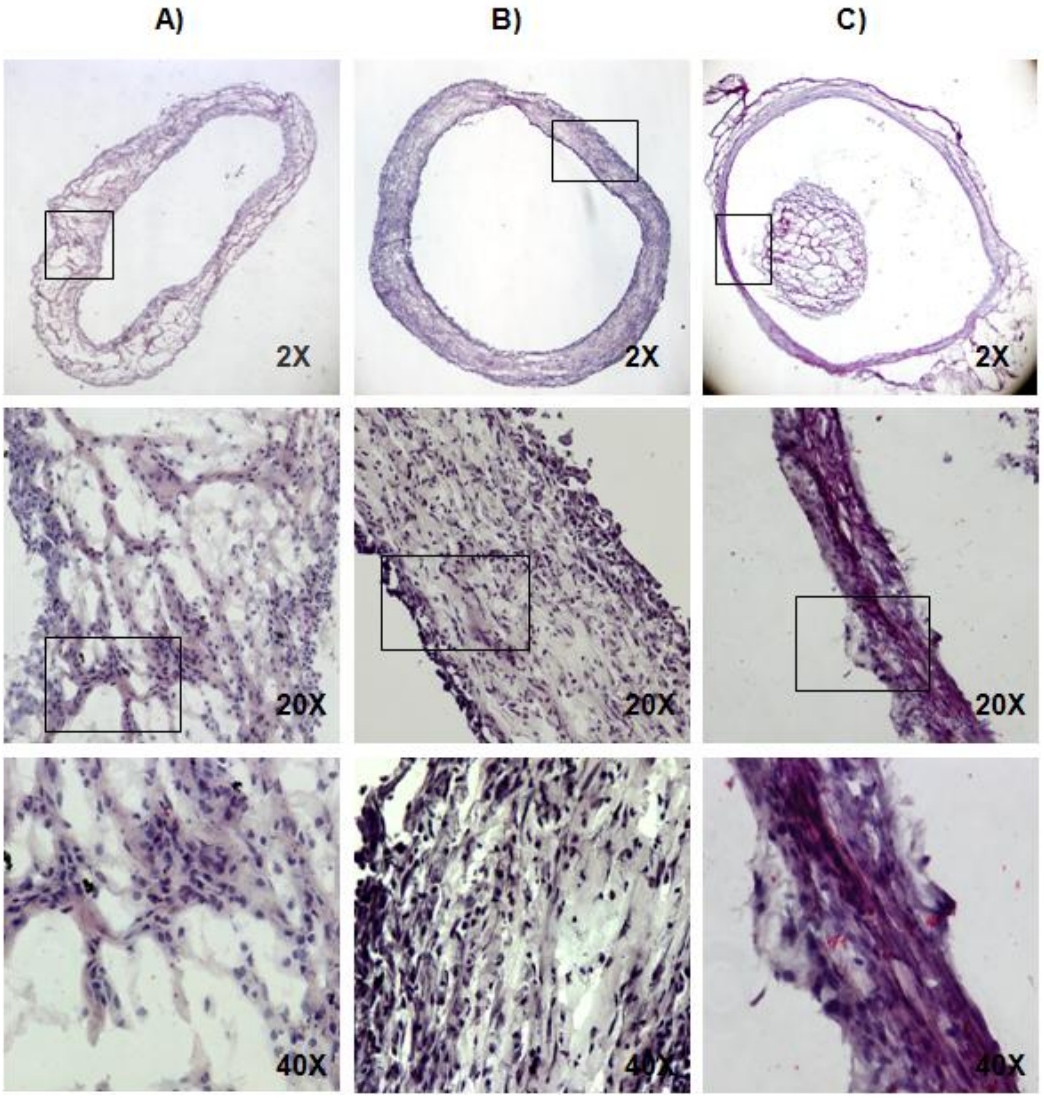


Figure 6-3. H & E staining of tube capsule histology of tissue capsule harvested from rat peritoneal cavity after 2 weeks.

In order to study the detailed structures of tissue capsule, the harvested tissue capsule (uncoated (A), fibronectin coated (B), fibronectin coated along with 50 million rSMSPC eGFP cells (C)) from rat's peritoneal cavity was sectioned and stained with haematoxylin to stain the nuclei, followed by eosin staining to stain other structures. The stained sections were examined by bright field microscope. Top panels show the complete structure of the tissue capsule with 2X magnification. Middle panels are magnified (20X) view of the area in top panel highlighted by black rectangle. The framed region in middle panel is further magnified to 40X from respective tissue capsule in the bottom panel. Data are representative of three independent experiments. n=3.

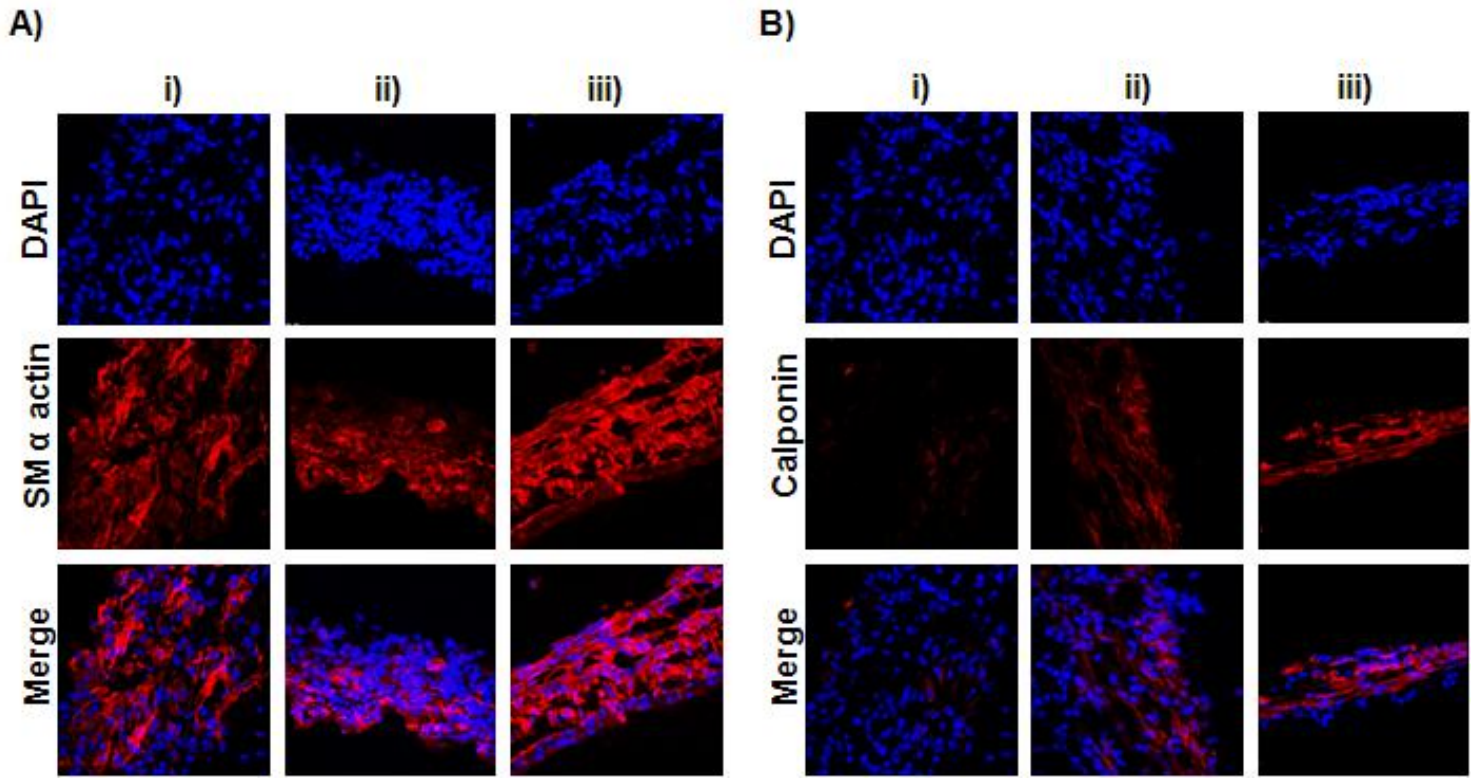


Figure 6-4. Immunofluorescence analysis of tissue capsule.
To investigate the role of smooth muscle cells in tissue capsule formation, the tissue capsule harvested from uncoated (i) fibronectin coated silastic tubes (ii) and fibronectin coated along with 50 million rSMSPC cells injected silastic tubes (iii) sections were immunostained for SM α actin (A) and calponin (B) antibodies. The immunostained sections were analysed in confocal microscope. The nuclei were stained with DAPI (blue) and smooth muscle markers SM α actin (Red) and calponin (Red) respectively. Results are representative of three independent experiments. n=3.

6.4. Discussion

The current experiments aimed to investigate the contribution of rSMSPC expressing eGFP cells to SMC content of tissue capsule formed in rat's peritoneal cavity. The results of this present study demonstrate that 1) presence of fibronectin alone or in combination with rSMSPC eGFP cells induced multiple layers of tissue encapsulation covering the entire length of tube. 2) Histological analysis confirmed the presence of tightly packed cells in fibronectin coated tubes injected with rSMSPC expressing eGFP cells. 3) Integration of rSMSPC eGFP cells within the tissue capsule was detected by both confocal and FACS analysis in fibronectin coated tube capsules injected with rSMSPC eGFP cells. Finally, expression of smooth muscle markers such as SM α actin and calponin were found to be relatively high in fibronectin coated tissue capsules injected with rSMSPC expressing eGFP cells. However, insufficient rSMSPC eGFP cell integration into neovessels *in vivo* was observed, suggesting that rSMSPC expressing eGFP cells contribute only a small extent to the SMC content of the tissue capsule. A possible explanation for this might be that these cells require more time to integrate into the capsule or require other cues such as cytokines, chemokines, cytoprotective factors or growth stimulators for more effective engraftment and expansion. Therefore, additional experiments will be required using varying cell number for injection and increasing the length of silastic tube inside the cavity might enhance SMSPC integration into capsule. Moreover, a number of additional growth promoters, cytokines such as TGF-β, IGF-1, and PDGF-BB would need to be tested with SMSPC cells and capsule formation *in vivo*.

Interestingly, tissue capsules isolated from the fibronectin coated tubes injected with rSMSPC eGFP cells did not collapse while suturing for OCT processing, suggesting that integration of small number of rSMSPC eGFP into capsule may enhance capsule structural integrity. It is also conceivable that SMSPC have exhibited ECM effects within the capsule as shown in other in vitro studies of smooth muscle progenitor cells [375]. Further gene or chromosome tagging studies would be required to examine whether implanted cells differentiated into SMC *in vivo* but remained undetected by our analysis as it is known that GFP can be down regulated following stem cell differentiation. It is also interesting to note that calponin staining was higher in SMSPC treated capsules suggesting possible augmentation SMC differentiation within capsule. This latter effect could conceivably have been mediated through paracrine factors secreted from implanted SMSPC. However,

further research will need to be carried out to investigate the specific role of rSMSPC eGFP cells in neovessel formation in the rat peritoneal cavity.

In addition, a wide variation in tissue capsule formation was observed between animals. This is most likely because of the small number of animals studied (n=3) per group. Thus, data reported in this study represent a preliminary step to investigate the rSMSPC differentiation into SMC in vitro. More experiments are required to verify the effect of rSMSPC eGFP cells on reducing the inflammatory responses in rat peritoneal cavity and augmenting more myofibroblasts differentiation to SMC phenotype. In addition, a further study is undertaken to investigate the role of rSMSPC eGFP in three dimensional (3D) tissue formation on biodegradable porous poly (L-lactic acid) (PLLA) scaffold *in vivo* in rats.

In summary, this study investigated the role of rSMSPC eGFP in tissue capsule formation and found that rSMSPC eGFP can integrate into tissue capsule structure *in vivo* and contribute to their smooth muscle cell content. It is conceivable that small number seeded SMSPCs may also have paracrine effects on modulating myofibroblasts within the capsule augmenting SMC differentiation through bystander effect. Thus, better understanding of rSMSPC differentiation into SMC may provide important developments in tissue engineering and may lead to autologous vascular grafts in using a patient's own cells as a source which mainly overcomes any immune response.

7. General Discussion and Future directions

General Discussion and Future directions

The goals of the present work were to investigate myocardin gene regulation involved in differentiation of hSMSPC into SMC. This study selected hSMSPC cells, a novel circulating *Isl*-1^{+} stem progenitor cell isolated from bypass filters as a cell model to evaluate SMC differentiation. SMSPC are clonogenic, self renewing with high population doubling and express classic stem cell markers such as Oct 4, Sox-2, Nanog, KLF4, SSEA-1 and c-kit.

The work has been divided into four parts. The first part characterised undifferentiated hSMSPC cells for the presence of smooth muscle transcription factors and their repressors, the second part identified putative binding sites for smooth muscle transcriptional repressors on the myocardin promoter region, the third part determined the molecular mechanism for smooth muscle differentiation in hSMSPC cells *in vitro* and finally, the fourth part investigated the integration of rSMSPC expressing eGFP into tissue capsule formation and their contribution to their smooth muscle content in a rat's peritoneal cavity *in vivo.*

In specific aim 1, hSMSPCs were characterised using qRT-PCR and found that the transcript levels of smooth muscle transcription factors, smooth muscle markers and smooth muscle repressors. hSMSPC cells express SRF, a major transcription factor required for SMC marker gene transcription, suggesting that hSMSPC cells may be already in a permissive state enabling differentiation towards smooth muscle cells. Notably, SRF alone is a weak transcriptional activator and it depends on a complex formation in a 2:1 stoichiometric ratio with its powerful coactivator, myocardin [94, 142, 143, 145] for initiation of SMC gene transcription [111, 112].

Myocardin is the major SRF cofactor that controls differentiation of smooth muscle cells [94, 142, 143, 145]. Furthermore, overexpression of myocardin alone is sufficient to induce CArG-SRF dependent SMC differentiation markers in a variety of cell types such as 10T1/2 cells, NIH 3T3 cells and 3T3-L1 cells [350]. Of note, myocardin homologous mutant mice die by embryonic day (E) 10.5 exhibiting no evidence for VSMC differentiation [158], indicating that myocardin is necessary for VSMC differentiation [143]. However, hSMSPC cells express low levels of myocardin gene expression. Therefore, it is important to test whether myocardin expression is critical for SMC differentiation in hSMSPC cells.

Furthermore, hSMSPCs express myocardin repressors including KLF4 and Elk-1, raising the possibility that presence of these repressors may be responsible for maintenance of stem progenitor phenotype by suppression of myocardin gene

expression in hSMSPC cells. Thus, hSMSPC cells are a useful model to study the regulation of myocardin signaling pathway involved in differentiation of progenitor cells into SMCs.

Examination of different growth conditions for induction of SMC differentiation indicated that SmGM2 with 1% FBS can induce SMC differentiation with an increase in SM α actin expression. However, SmGM2 1%FBS media is not sufficient to induce full repertoire of SMC marker genes. TGF β1 signalling controls SMC differentiation in embryonic stem cells and neural crest [239, 240]. TGF-β1 induces SMC differentiation by binding to three TCE elements (CArG box, SBE and TCE elements) located within the promoters of smooth muscle markers [239]. Investigation of smooth muscle associated cytokines to induce SMC differentiation in hSMSPC cells showed that TGF-β1 can induce the expression of SM α actin and calponin to a lesser extent in hSMSPC cells, suggesting that the effect of TGF- β1 may be inhibited by transcriptional repressors in hSMSPC cells.

Multiple repressors are involved in the downregulation of myocardin induced SMC transcription [164]. Passmann *et al.,* [302] demonstrated that downregulation of SRF and myocardin by KLF4 is responsible for progenitor phenotype maintenance in $Sca1^{+}$ vascular SMC progenitor cells isolated from adventitia of mice. Already, KLF4 has been identified as a negative regulator for myocardin induced SMC differentiation [138, 139, 167]. Thus, it was hypothesised that KLF4 is inhibiting myocardin gene expression in hSMSPC cells.

A search for putative binding site for transcriptional repressors within 3.903 Kb of the myocardin promoter region resulted in the identification of five potential KLF4 binding sites within the promoter region with high similarity scores (Fig.4.5). It may be possible that KLF4 binding within these predicted binding sites is responsible for KLF4 mediated repression of myocardin gene expression in hSMSPC cells. The possibility of KLF4 binding within the human myocardin promoter in hSMSPC has been verified using qRT-PCR following the ChIP assay. However, there are limitations to use of the qRT-PCR assay as a standalone technique to confirm the transcription factors binding within the promoters, due to its inevitable errors during the amplification process. Therefore, it requires more specific additional studies such as EMSA and site directed mutational analysis to further strengthen the direct binding of KLF4 within the human myocardin promoter in hSMSPCs. KLF4 is involved in the phenotypic switching of SMCs upon various stimuli such as PDGF-BB, oxidised phospholipids and vascular injury. Hence, identification of direct KLF4 binding within the myocardin promoter sheds light on a

better understanding of molecular mechanisms that regulate myocardin gene expression. Further development of small molecules to block the binding of KLF4 within the myocardin promoter will allow mechanistic studies of KLF4 contributing to SMC phenotype switching during development of atherosclerosis.

The human myocardin promoter activity was studied using a lentiviral construct consisting of a human myocardin promoter driving eGFP expression in different cell types and found that the myocardin promoter can specifically activate eGFP expression in hSMSPC and in HASMC, but not in HUVEC cells, suggesting that basic SMC transcriptional machinery exists within hSMSPC. To test whether knockdown of KLF4 upregulates myocardin gene expression in response to TGF-β1 stimulation, the hSMSPC cells were cotransduced with both of LV_shKLF4 B lentiviral supernatant and HR-ChMYOCDGW constructs in differentiation media. Results from the cotransduction assay showed that TGF-β1 stimulation increases myocardin promoter activity, indicating that myocardin transcriptional machinery is repressed by the presence of KLF4 in hSMSPC cells. Although, KLF4 has been reported to have repressor function via physical association with the myocardin/SRF complex [167], there currently exists no direct evidence for KLF4 transcription regulation of myocardin gene expression. Based on these results, it was hypothesised that KLF4 may physically bind to the human myocardin promoter region in hSMSPC cells and repress its expression.

Binding of KLF4 within human myocardin promoter was verified by ChIP assay in hSMSPC cells. The result from KLF4 ChIP assay in hSMSPC strongly confirmed for the first time, that KLF4 binds within the human myocardin promoter *in vitro*. This study will need to be expanded to determine the roles of the five putative KLF4 binding sites on basal and growth factor induced increase in myocardin gene expression in hSMSPC cells.

To study the functional role of TGF-β1 stimulation on hSMSPC cells that have undergone KLF4 knockdown, expression levels of KLF4 were silenced using LV_shKLF4 B and subsequently stimulated with TGF-β1. Specific knockdown of KLF4 in hSMSPC resulted in loss of their progenitor phenotype and adoption of an elongated morphology. Results from TGF-β1 stimulation on KLF4 knockdown indicate that TGF-β1 induced SMC differentiation with an increase in SM α actin and calponin expression in hSMSPC cells. These data confirm that KLF4 plays a major role in maintenance of hSMSPC progenitor phenotype and differentiated phenotype repression is mediated by transcriptional suppression of the myocardin gene. This result is consistent with previously reported findings showing that KLF4 plays a

major role in repression of myocardin induced adult SMC differentiation [167]. In these latter cultured cells, PDGF-BB stimulation induced expression of KLF4 which in turn decreased SRF binding within the CArG elements and repressed myocardin induced SMC gene transcription [167]. This current study identified a direct KLF4 binding within the myocardin promoter which supports and strengthens the previous findings through identification of an additional mechanism through which KLF4 mediates the repression of myocardin gene regulation by direct physical interaction with the myocardin promoter.

KLF4 is highly expressed in ESC and plays a major role in maintenance of embryonic stem cell pluripotency [376]**.** KLF4 regulates the expression of core transcription factors of pluripotency including Oct4, Sox2 and Nanog. The promoters of each of these core transcription factors have binding sites for all transcription factors and act in a regulatory circuitry with a high degree of autoregulation between them. However, the KLF4 promoter has no binding sites for Oct4, Sox2 or Nanog genes and KLF4 is exempt from this core regulatory activity. These studies suggest that KLF4 is a major regulator for ESC core transcription factors. Therefore, downregulation of KLF4 is critical for differentiation of stem cells [338]. Recent evidence demonstrates that KLF2 and KLF5 genes are also involved in the regulation of the pluripotency transcription factors [376]. Several recent reports showed that downregulation of all three KLFs can induce extensive differentiation in ESC [376]. Therefore, experiments in the current work should be further expanded to analyse the effect of triple knockdown (KLF2\KLF4\KLF5) in induction of SMC differentiation in hSMSPC cells. Interestingly, there is already preliminary evidence from our group showing binding of KLF5 to the repressor regions within the human myocardin promoter in hSMSPC cells (Libby Turner, 2013, personal communication).

To determine whether overexpression of myocardin can induce SMC differentiation, hSMSPC cells were transduced with a lentiviral vector encoding the myocardin gene. Results showed that overexpression of myocardin significantly increased the stellate cell formation with spindle morphology and induced SMC differentiation with expression of myofilamentous SM markers including SM α actin, calponin and SMMHC in hSMSPC cells. In addition, nearly 80% of the myocardin transduced cells were induced to differentiate into myofilamentous and contractile like SMCs, which is consistent with other findings showing that overexpression of myocardin is sufficient to induce smooth muscle differentiation in various cell types including 10T1/2, NIH3T3 and 3T3 L1 cells [350]. Taken together, these data

confirm that myocardin gene expression plays a pivotal role in hSMSPC differentiation into SMC. It would be interesting to extend these findings to determine contractile function of differentiated hSMSPC cells using calcium signalling and angiotensin II responsiveness as described by Simper *et al*.,[245] and [377].

hSMSPC cells from a regenerative medicine perspective are attractive cells since they have an unlimited self renewal capability *ex vivo*, which can be expanded outside the body to potentially unlimited quantities. To investigate whether rat SMSPC cells expressing eGFP have an ability to integrate into tissue capsules formed in the rat peritoneal cavity *in vivo* and contribute to their smooth muscle content, hSMSPCs were injected into peritoneal cavity of rat along with silastic tubes. rSMSPC eGFP cells integrated into the tissue capsule structure but with low level of efficiency (~1%). Moreover SMSPC treated capsules appeared to contain myofibroblasts with more contractile calponin positive immunophenotype compared to non cell seed controls. However, this model showed a huge variation in tube capsule formation. A possible explanation for this might be due to small numbers of animals (n=3) per group. It would be interesting to further extend this work with larger numbers of animals per group. Moreover, this model needs to be tested with varying numbers of cells, augmenting factors such as chemokines, cytokines, chemoattractants, growth factors and differentiation agents.

Experiments are currently underway to evaluate the *in vivo* differentiation potential of rSMSPC cells to determine whether these cells have the ex vivo ability to populate bio matrix-vascular grafts in rats. These studies use biodegradable porous 3D poly (L-lactic acid) (PLLA) scaffold seeded with cells prior to implantation into artery. Initial preliminary data supports extensive seeding of SMC derived from SMSPC in PLLA tubes out to 3 months post implantation as an interposition graft in rats (CL- personal communication). Extensive experiments are underway and full extent of SMC (derived from SMSPC) engraftment will be verified by immunofluorescence analysis for smooth muscle markers and tracer genetic studies. However, this study faces some significant challenges, including: how to clearly track and identify the undifferentiated rSMSPC cells *in vivo*? How to eliminate the undifferentiated cells *in vivo* to avoid the formation of teratoma? This latter concern is based on data showing that hSMSPC induce teratoma in nude rats (Caplice *et al*., unpublished data).

A recent study demonstrated that the introduction of a stem cell specific suicide gene including thymidine kinase (tk) into stem cell in combination with Ganciclovir (Gan) treatment was effective in killing undifferentiated stem cells [291].

However, the suicide gene approach required genetic manipulation of stem cells, which may cause tumorigenicity in animals [291]. Moreover this approach may activate the immune system which is counter regulatory in vascular grafting. Another recent study reported the use of cytotoxic antibodies directed against cell surface antigens such as SSEA-4 [291], or podocalyxin-like protein-1 [378] in hESCs. However, so far, too little is known about the specificity and side effects of these agents. Additional purification of undifferentiated and differentiated populations of SMSPC and SMC respectively may be achieved by detection of unique FACS profiles for these cells and area that is currently being extensively investigated by our group. Finally a more complete understanding of the molecular mechanisms involved in controlling hSMSPC stemness, self renewal and proliferation will enable advances in the development of techniques to control fate determination of undifferentiated SMSPC cells with consequences for safer therapeutic application.

In conclusion, this work has delineated the myocardin signaling pathways involved in SMC differentiation of hSMSPC cells. This is the first study to report that KLF4 acts as a transcriptional repressor of myocardin gene expression in hSMSPC cells mediated through direct binding to the myocardin promoter and requiring a consensus sequence at (-3702) on (+) the strand of TSS. Knockdown of KLF4 resulted in an increase of the myocardin promoter directed reporter genes and in combination with TGF-β1 stimulation, induced contractile-like SMC differentiation in hSMSPC cells. Moreover, overexpression of myocardin gene alone induced SMC differentiation in hSMSPC cells. Of interest, a small number of rSMSPC eGFP cells integrated into tube capsules grown within rat peritoneal cavity. Further studies will be necessary to determine whether the KLF4 knockdown alone or in combination with myocardin gene overexpression in hSMSPC is necessary and sufficient to generate large numbers of stably differentiated smooth muscle cells that can be manipulated for use in tissue engineered vascular grafts (an area of intense research and great clinical need within cardiovascular field).

8. Limitations of this study

The major limitation of this study comes from the use of passaged hSMSPC cells isolated from single patients instead from different patients. Therefore, it would be interesting to verify the differentiation potential of hSMSPC cells isolated from different patients.

Another limitation of this study is that the sequencing of cloned 3.925Kb fragment has detected one point mutation within the human myocardin promoter. These mutations may have affected transcription factor binding within the human myocardin promoter. Hence, the promoter mutation and their contribution to transcription factor binding should be tested in future experiments

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10. Appendix

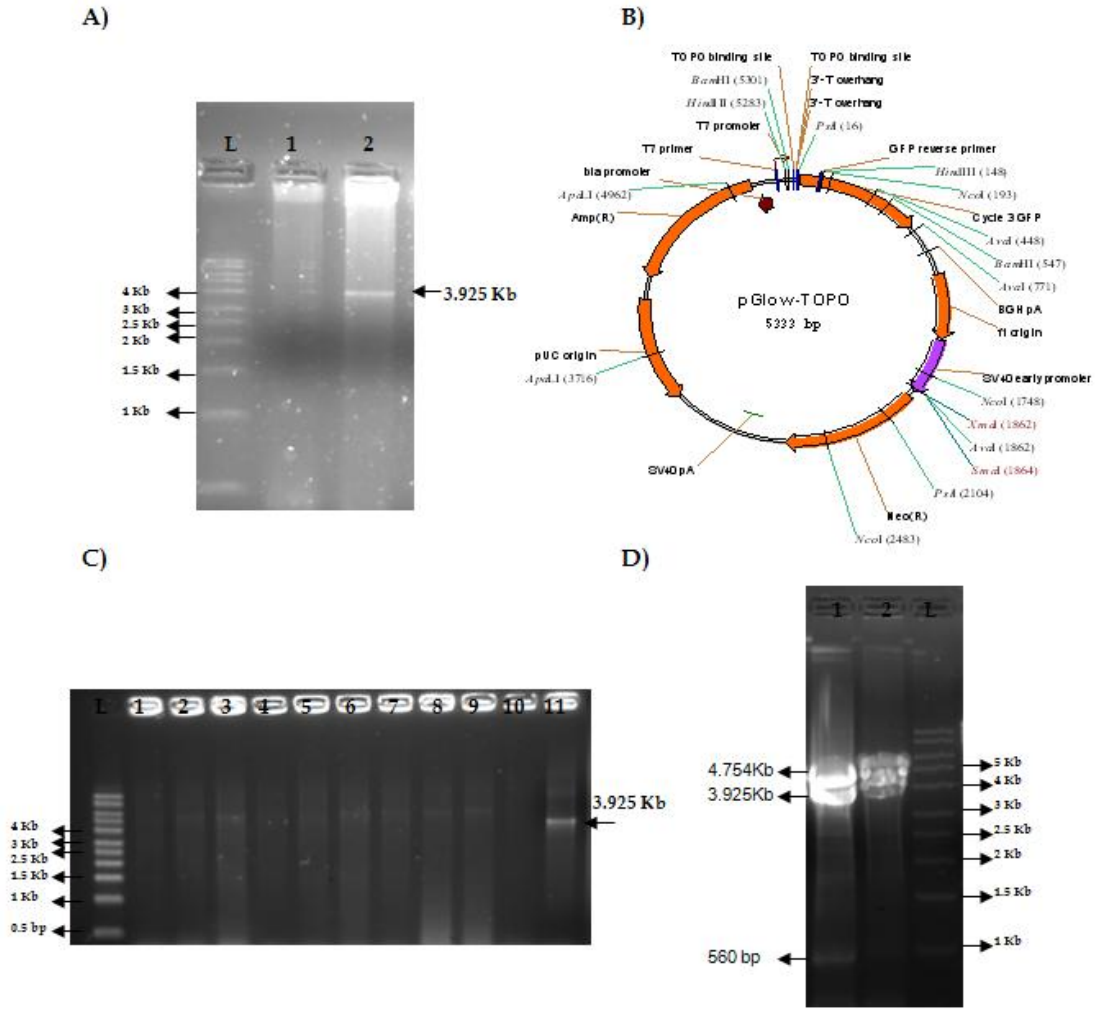


Figure 10-1. PCR amplification and TA cloning of 3.925 Kb human myocardin promoter regions from human genomic DNA.

A) PCR amplification of human myocardin promoter. Lane L- Sigma 1 Kb ladder, lane 2 is PCR amplified 3.925 kb human myocardin promoter region. B) TA cloning vector –pGlow TOPO vector map. C) Electrophoresis of colony PCR screening of transformed *E.coli* colony 1-11 to identify human myocardin promoter positive clone. Colony 11 is positive for human myocardin promoter. D) Agarose gel electrophoresis of recombinant plasmid pGlow TOPO after restriction with *EcoRI/BamHI* restriction enzymes on 1% agarose gel. A fragment size of 3.925 Kb in lane 1, 2 and 3 confirms the presence of human myocardin promoter within the recombinant pGlow TOPO plasmid. Lane L- Sigma 1Kb ladder.

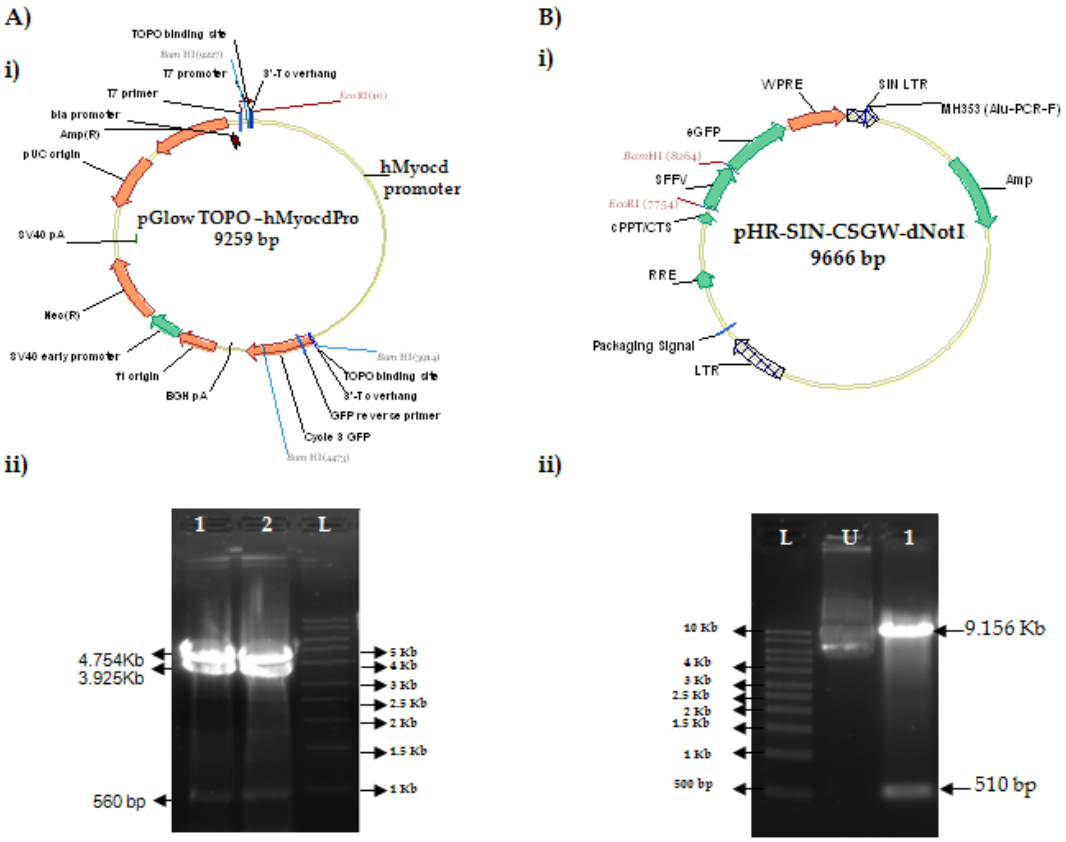


Figure 10-2. Sub-cloning of human myocardin promoter (3.925 kb) into HR-CSGW.

A) Insert preparation. i) Circular map of the 9259 bp recombinant pGlow TOPO plasmid containing 3.925 Kb human myocardin promoter. ii) Electrophoresis of the recombinant pGlow TOPO plasmid after restriction digestion with *EcoRI/BamHI* restriction enzymes (Lane 1, 2 and 3). The 3.925 Kb fragment was excised, purified and used as insert. Lane L- Sigma 1 Kb ladder. B) Vector preparation. i) Circular map of the 9666bp pHRSIN-CSGW-dNotI plasmid (HR-CSGW). ii) Electrophoresis of the plasmid HR-CSGW (lane 1) after restriction digestion with *EcoRI/BamHI* restriction enzymes showing fragment sizes of 9.1 Kb and 510 bp, vector backbone and the spleen focus-forming virus (SFFV) promoter, respectively. Lane L- Sigma 1Kb ladder, U-uncut plasmid**.**

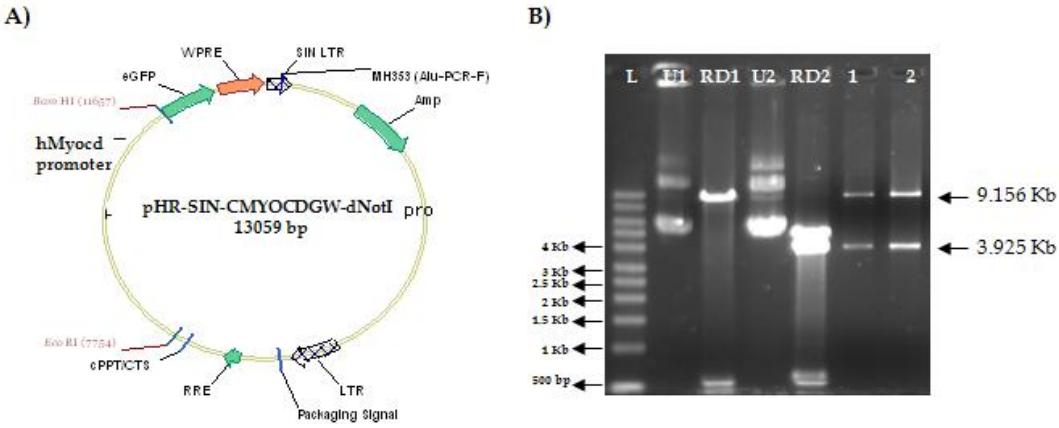


Figure 10-3. Sub-cloning of human myocardin promoter (3.925 kb) into HR-CSGW.

A) Circular map of the 13059 bp recombinant plasmid pHRSIN-CMYOCDGW (HR-CMYOCDGW) The 9.1Kb HR-CSGW vector backbone was ligated with gel purified 3.925 Kb human myocardin promoter insert to yield pHRSIN-CMYOCDGW-dNotI (HR-CMYOCDGW) plasmid. B). Electrophoresis of the recombinant plasmid HR-CMYOCDGW (lane 1 and 2) after restriction digestion with *EcoRI/BamHI* restriction enzymes showing fragment sizes of 9.1 Kb and 3.925 Kb, vector backbone and the human myocardin promoter, respectively. Lane L- Sigma 1Kb ladder, U1-uncut HR-CSGW plasmid, RD1- restriction digestion of HR-CSGW plasmid with *EcoRI/BamHI* restriction enzymes, U2- Uncut recombinant pGlow TOPO plasmid containing human myocardin promoter. RD2- restriction digestion of recombinant pGlow TOPO plasmid with *EcoRI/BamHI* restriction enzymes.

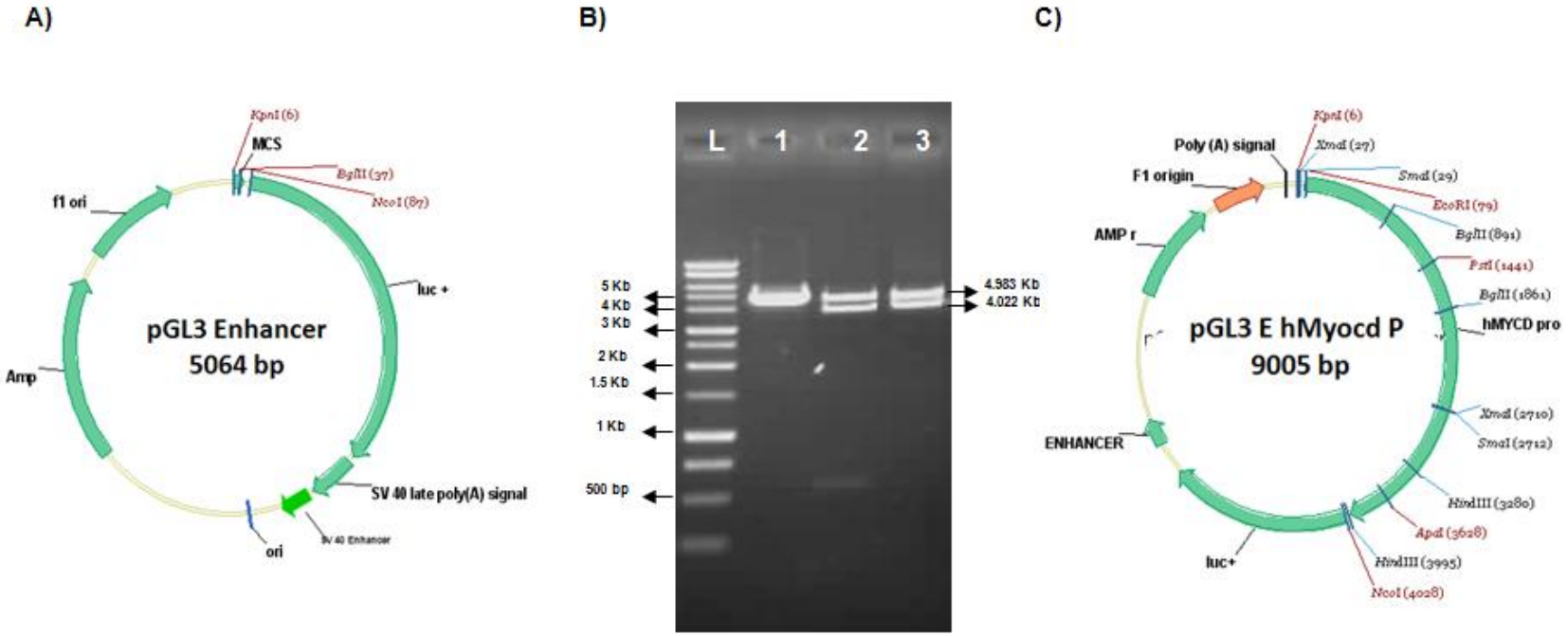


Figure 10-4. Sub-cloning of human myocardin promoter (3.925 kb) into pGL3 Enhancer vector.

A) Circular map of the 5064 bp plasmid pGL3 enhancer. B). Electrophoresis of the recombinant plasmid pGL3 E hMyocd P plasmid. The 5.064 Kb *BglII* linearised pGL3 enhancer vector backbone (lane 1) was ligated with gel purified 3.925 Kb human myocardin promoter insert (lane 2) from pGlow TOPO hmyocd-promoter plasmid to yield pGL3E hMyocdP plasmid (lane 3). lane L – 1Kb Promega ladder, lane 1 pGL3 Basic vector cut with *BglII,* Lane 2 pGlow TOPO + hMyocd promoter cut with *BamHI,* lane 3 recombinant plasmid cut with *KpnI/NcoI. C)* Circular map of the 9005 bp recombinant pGL3 E hMyocd P plasmid.

A)

>hMyocardin Promoter (3.925 kb)

AATTCTATCAATAACCAGTGAACTTGGAAGGGGACACTGAGCTTCTCA
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G

D)
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Figure 10-5. Sequencing of 3.925 Kb cloned human myocardin promoter .

A) 3.925 Kb length of the human myocardin promoter was extracted from human genome using *in silico* PCR B) Seq-1- Forward sequence obtained from sequencing of cloned human myocardin promoter. C) From the forward sequence, the next primer was designed to sequence the next segment of cloned myocardin promoter. The sequence was named as Seq2. D) Seq 2 was used for designing primer to obtain seq3 and E) Seq3 was used as reference sequence for designing primer to get the last segment of cloned myocardin promoter sequences and named as Seq4.

A)

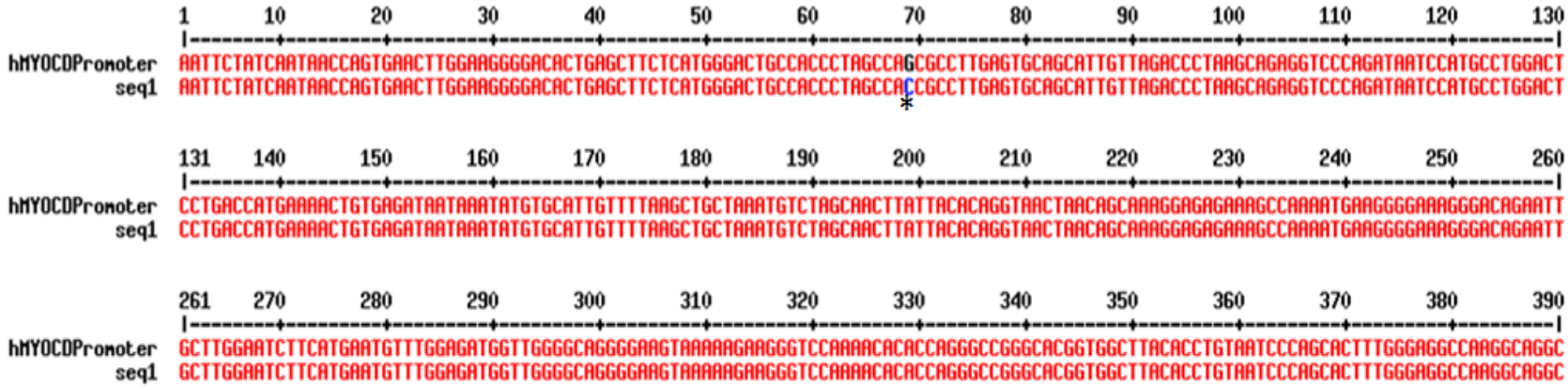


Figure 10-6. Alignment of sequences obtained from sequencing of cloned human myocardin promoter against 3.925 Kb human myocardin promoter extracted from human genome using *in silico* PCR.

A) The results showed one mismatch within the seq1 sequence. The mismatch was highlighted as (*) mark within the aligned sequence

Table 10-1. List of transcription factors binding within 3.9 Kb human myocardin promoter

		Position				
Sno	Matrix	from	to	anchor	Core sim	CAPITALS:core sequence
1	V$HLF.01	2268	2284	2276	1.000	agcgattacGTAAcatc
2	V$ACAAT.01	1605	1619	1612	1.000	cccaCCAAtctcttc
3	V$LYF1.01	3485	3497	3491	1.000	atcTGGGAgaggg
4	V$TGIF.01	3634	3650	3642	1.000	gagcgcctGTCAgtagt
5	V$TFII-IR4.01	1823	1833	1828	1.000	tggGATTagga
6	V$TGIF.01	1170	1186	1178	1.000	ggatatatGTCAgtagc
7	V$MZF1.02	241	251	246	1.000	aaGGGGaaagg
8	V$MASH1.01	3413	3425	3419	1.000	gaaccacCTGCaa
9	V$MZF1.01	1967	1977	1972	1.000	gtGGGGaaggg
10	V$KAISO.01	2249	2259	2254	1.000	attcCTGCtat
11	V$E2F.02	3270	3286	3278	1.000	aggctgcgcGAAAgggc
12	V$MZF1.02	2101	2111	2106	1.000	taGGGGaaaat
13	V$NFY.02	3621	3635	3628	1.000	tcagCCAAtcaggag
14	V$TGIF.01	2376	2392	2384	1.000	gctgatgtGTCAgagac
15	V$GATA.01	737	749	743	1.000	tacaGATAagacg
16	V$MZF1.02	2824	2834	2829	1.000	taGGGGaaaat
17	V$NGN_NEUROD.01	2560	2572	2566	1.000	tctcCATCtgtca
18	V$ZKSCAN3.01	1958	1980	1969	1.000	gctcccttCCCCaccttctgctt
19	V$SPI1_PU1.02	294	314	304	1.000	ggggcaggGGAAgtaaaaaga
20	V$TBX5.01	2136	2156	2146	1.000	tcaaaatcaaGGTGtcagcgg
21	V$HOXB5.01	3802	3820	3811	1.000	agagtTAATtagccccgca
22	V$GSH2.02	3800	3818	3809	1.000	cggggctAATTaactctta
23	V$MZF1.02	3171	3181	3176	1.000	gaGGGGaatgc
24	V$ZF5.01	1314	1324	1319	1.000	gtgcGCGCgtg
25	V$ZF5.01	3259	3269	3264	1.000	gtgcGCGCccg
26	V$S8.01	3797	3817	3807	1.000	ggggcTAATtaactcttagcc
27	V$ZF5.01	1316	1326	1321	1.000	gtgtGCGCgcg
28	V$S8.01	2659	2679	2669	1.000	gtataTAATtagggcttattt
29	V$CABL.01	1063	1073	1068	1.000	aaAACAaaaaa
30	V$DLX1.02	2193	2211	2202	1.000	acagcaagatAATTaatgt
31	V$S8.01	2197	2217	2207	1.000	caagaTAATtaatgtgtgctg
32	V$S8.01	3802	3822	3812	1.000	agagtTAATtagccccgcacg
33	V$MZF1.01	3041	3051	3046	1.000	ggGGGGacaaa
34	V$MYT1.02	2854	2866	2860	1.000	gcaAAGTtttga
35	V$MZF1.01	3827	3837	3832	1.000	ggGGGGaggcg
36	V$TBX5.01	2771	2791	2781	1.000	atctggccgaGGTGttatagg
37	V$MZF1.02	438	448	443	1.000	agGGGGaagcc
38	V$HNF3.01	1264	1280	1272	1.000	tccctgcAAACacacat
39	V$NFAT.01	3444	3462	3453	1.000	ccggaGGAAagaaggaagg
40	V$SOX5.01	74	96	85	1.000	gggtctaaCAATgctgcactcaa
41	V$SALL1.01	625	637	631	1.000	taATAAaaaataa
42	V$SMARCA3.02	1076	1086	1081	1.000	aagcACTTttg
43	V$MEL1.02	1876	1892	1884	1.000	ggcagtgGATGagatta
44	V$IK2.01	1229	1241	1235	1.000	gtttGGGAaatgc
45	V$YY1.02	2022	2042	2032	1.000	ttcctCCATttttgaagccag
46	V$MEL1.02	1386	1402	1394	1.000	tgacctaGATGagctgg
47	V$S8.01	2192	2212	2202	1.000	cacatTAATtatcttgctgtt

48	V$HOXA3.01	3799	3817	3808	1.000	ggggcTAATtaactcttag
49	V$GATA1.06	445	457	451	1.000	tagaGATAgggct
50	V$S8.01	2664	2684	2674	1.000	agcccTAATtatatacgtata
51	V$HSF2.02	2358	2382	2370	1.000	cagagacgaggaAGAAcaatccatc
52	V$CDX1.01	633	651	642	1.000	tgtgtttTTTAttattatt
53	V$DELTAEF1.01	391	403	397	1.000	agatcACCTgagc
54	V$MOK2.02	59	79	69	1.000	accctagccagcgCCTTgagt
55	V$CMYC.01	2636	2648	2642	1.000	ggtccaCGTGcat
56	V$CDX1.01	620	638	629	1.000	attatttTTTAttattttg
57	V$HNF3B.01	710	726	718	1.000	tggaagtaAATAtttct
58	V$SOX5.01	156	178	167	1.000	gcttaaaaCAATgcacatattta
59	V$RU49.01	1626	1632	1629	1.000	gAGTAcc
60	V$IK2.01	1688	1700	1694	1.000	gtttGGGAacggg
61	V$USF.01	2637	2649	2643	1.000	tatgCACGtggac
62	V$GATA1.01	431	443	437	1.000	tcttGATAggggg
63	V$DLX1.02	3803	3821	3812	1.000	gtgcggggctAATTaactc
64	V$ILF1.01	1362	1378	1370	1.000	agagagaaAACAactta
65	V$ILF1.01	906	922	914	1.000	gcaaggaaAACAtactc
66	V$ILF1.01	2475	2491	2483	1.000	ttgctgaaAACAcatta
67	V$RU49.01	2056	2062	2059	1.000	aAGTAct
68	V$AHRARNT.03	3353	3377	3365	1.000	ctggggatggGCGTgcgcagccggg
69	V$BSX.01	3800	3818	3809	1.000	taagagttAATTagccccg
70	V$DLX1.01	2665	2683	2674	1.000	atacgtatatAATTagggc
71	V$HSF2.02	809	833	821	1.000	gcctccagtgtgAGAAagatcttct
72	V$ILF1.01	638	654	646	1.000	taataaaaAACAcacca
73	V$HSF2.02	1766	1790	1778	1.000	ggtagaagagaaAGAAagatctcag
74	V$HNF3.01	2652	2668	2660	0.971	aagttgcAAATaagccc
75	V$VMYB.04	2718	2730	2724	1.000	tttAACGggcttc
76	V$SALL1.01	638	650	644	1.000	taATAAaaaacac
77	V$NGN_NEUROD.01	489	501	495	1.000	catgCATCtgtaa
78	V$STAT3.02	700	718	709	1.000	ttacTTCCaggaagagttg
79	V$HOXA3.01	2194	2212	2203	1.000	cacatTAATtatcttgctg
80	V$ZFX.01	2323	2333	2328	1.000	ctGGCCtgggc
81	V$DLX1.02	3798	3816	3807	1.000	gctaagagttAATTagccc
82	V$PTX1.01	1821	1837	1829	1.000	tgtcCTAAtcccaggaa
83	V$STAT3.02	702	720	711	1.000	actcTTCCtggaagtaaat
84	V$DLX2.01	2198	2216	2207	1.000	agcacacattAATTatctt
85	V$DEC2.01	2635	2649	2642	1.000	tatgcaCGTGgaccc
86	V$NGN_NEUROD.01	2784	2796	2790	1.000	tgtgCATCtggcc
87	V$DLX2.01	2660	2678	2669	1.000	aataagccctAATTatata
88	V$BSX.01	3801	3819	3810	1.000	gcggggctAATTaactctt
89	V$GATA3.01	147	159	153	1.000	gtgaGATAataaa
90	V$AREB6.02	349	361	355	1.000	cttaCACCtgtaa
91	V$SOX5.01	2352	2374	2363	1.000	aggaagaaCAATccatccatgtt
92	V$IK2.01	2866	2878	2872	1.000	gcttGGGAaggct
93	V$RORA1.01	1389	1411	1400	1.000	gctcatctaGGTCatgcagaccc
94	V$IPF1.01	3798	3816	3807	1.000	gctaagagtTAATtagccc
95	V$SOX5.01	646	668	657	1.000	gcccagaaCAATtttggtgtgtt
96	O$XCPE1.01	3514	3524	3519	1.000	ggGCGGgagca
97	V$GSH2.02	3801	3819	3810	1.000	aagagttAATTagccccgc
98	V$KAISO.01	939	949	944	1.000	tgtcCTGCaat
99	V$PAX4.02	3803	3817	3810	1.000	gagttAATTagcccc

100	V$NMP4.01	1528	1538	1533	1.000	agAAAAagcca
101	V$DEC2.01	2636	2650	2643	1.000	ggtccaCGTGcatat
102	V$NFAT.01	2088	2106	2097	1.000	ggtgaGGAAatcgattttc
103	V$SIX1.01	3650	3664	3657	1.000	taaaggTATCagat
104	V$ZBP89.01	3820	3842	3831	1.000	actggcgcctCCCCctcgccgt
105	V$PTX1.01	2874	2890	2882	1.000	caagCTAAgcccagccc
106	V$YB1.01	3624	3636	3630	1.000	ctgatTGGCtgag
107	**V$GKLF.01**	**3439**	**3455**	**3447**	**1.000**	**aaagaaggaAGGGctgc**
108	V$YY2.01	726	746	736	1.000	taactCCATtttacagataag
109	V$NFKAPPAB65.01	2819	2831	2825	1.000	aatggattTTCCc
110	V$SOX9.02	835	857	846	1.000	atgtgacACAAaggcaagacatg
111	V$CABL.01	2742	2752	2747	1.000	aaAACAaagac
112	V$AHRARNT.03	3257	3281	3269	1.000	gcgcgaaaggGCGTgcgcgcccgcg
113	V$CDPCR3HD.01	2754	2772	2763	1.000	tttcattGATCcatgtgcc
114	V$MAFA.01	966	986	976	1.000	cgcTGCTgcattagcctgctg
115	V$BSX.01	2196	2214	2205	1.000	cacacattAATTatcttgc
116	V$MEIS1.01	2561	2577	2569	1.000	ctccatcTGTCaccgac
117	V$LMX1B.01	2198	2220	2209	1.000	aaacagcacacatTAATtatctt
118	V$FTF.01	919	931	925	1.000	actcCAAGgtggt
119	V$VAX1.01	2196	2214	2205	1.000	gcaagatAATTaatgtgtg
120	V$ZNF35.01	2365	2377	2371	1.000	acgaggAAGAaca
121	V$SATB1.01	633	647	640	1.000	aatAATAataaaaaa
122	V$BSX.01	2195	2213	2204	1.000	agcaagatAATTaatgtgt
123	V$AREB6.01	202	214	208	1.000	tagttACCTgtgt
124	V$PAX4.02	3802	3816	3809	1.000	gggctAATTaactct
125	V$NANOG.01	2966	2984	2975	1.000	ccgagggAATGggaaaaga
126	V$RORA1.01	880	902	891	1.000	ggaaaagaaGGTCatggagtagc
127	V$ETS1.01	2031	2051	2041	1.000	aaaatggaGGAAgcggccatg
128	V$CEBPB.01	1583	1597	1590	1.000	ataatttGCAAtat
129	V$ZNF35.01	701	713	707	1.000	tccaggAAGAgtt
130	V$SMARCA3.01	3036	3046	3041	0.960	tcACATttgtc
131	V$GATA1.03	1578	1590	1584	1.000	ctcaGATAatttt
132	V$SMARCA3.01	1171	1181	1176	0.960	tgACATatatc
133	V$SMARCA3.01	3081	3091	3086	1.000	ttCCATttccc
134	V$IPF1.01	3803	3821	3812	1.000	gtgcggggcTAATtaactc
135	V$ZF5.01	3261	3271	3266	1.000	gcgtGCGCgcc
136	V$TEF.01	2267	2283	2275	1.000	tgatgttacGTAAtcgc
137	**V$GKLF.02**	**216**	**232**	**224**	**1.000**	**cagcAAAGgagagaaag**
138	V$CEBPB.01	1229	1243	1236	1.000	gagtttggGAAAtgc
139	V$CEBPB.01	2268	2282	2275	0.940	gatgttacGTAAtcg
140	V$CMYB.02	2805	2817	2811	1.000	cgTAACcgaccca
141	V$FAC1.01	641	651	646	1.000	taaaaAACAca
142	V$SATB1.01	623	637	630	1.000	aatAATAaaaaataa
143	V$HOXC8.01	2197	2215	2206	1.000	caagataATTAatgtgtgc
144	V$GATA1.03	108	120	114	1.000	cccaGATAatcca
145	V$CDP.02	3618	3636	3627	1.000	ctcagcCAATcaggagcgg
146	V$MSX2.01	2662	2680	2671	1.000	taagccCTAAttatatacg
147	V$HSF2.02	3122	3146	3134	1.000	gctgcgagatacAGAAtcgtctctg
148	V$ARNT.01	2634	2650	2642	1.000	ggggtccaCGTGcatat
149	V$BARX2.01	2663	2681	2672	1.000	acgtataTAATtagggctt
150	V$NF1.03	1223	1243	1233	1.000	gagtttgggaaatGCCAataa
151	V$SPI1_PU1.02	1963	1983	1973	1.000	gaaggtggGGAAgggagcatt

152	V$LMX1B.01	622	644	633	1.000	aaataataaaaaaTAATaataaa
153	V$E4BP4.01	2265	2285	2275	1.000	cagcgattacGTAAcatcatt
154	V$GATA3.02	1779	1791	1785	1.000	gaaAGATctcagg
155	V$CRX.01	1881	1897	1889	1.000	ctcccTAATctcatcca
156	V$HIC1.01	3458	3470	3464	1.000	gcggaTGCCcgga
157	V$CEBPA.01	1553	1567	1560	1.000	tattttgaGCAAcat
158	V$ELF2.01	697	717	707	1.000	tacttccaGGAAgagttggga
159	V$GSH2.01	1428	1446	1437	1.000	ttcagcTAATttcatccca
160	V$DEC2.01	2760	2774	2767	0.903	taggcaCATGgatca
161	V$SMARCA3.01	1479	1489	1484	1.000	tgCCATgtatc
162	V$GSH2.01	2469	2487	2478	1.000	cccctcTAATgtgttttca
163	V$ZNF219.01	3817	3839	3828	1.000	ggcgcctCCCCcctcgccgtgcg
164	V$MEL1.03	1024	1040	1032	1.000	tggagaaGATGttctca
165	V$NANOG.01	2250	2268	2259	1.000	tagcaggAATGggaaaatg
166	V$ARNT.01	2635	2651	2643	1.000	catatgcaCGTGgaccc
167	V$SPI1_PU1.02	434	454	444	1.000	tgatagggGGAAgccctatct
168	V$FAC1.01	319	329	324	1.000	tccaaAACAca
169	V$MAZ.01	1803	1815	1809	1.000	atggGAGGagagg
170	V$RBPJK.02	2255	2267	2261	1.000	ggaaTGGGaaaat
171	V$ZF5.01	3258	3268	3263	1.000	gcggGCGCgca
172	V$MAZ.01	3821	3833	3827	1.000	cggcGAGGgggga
173	V$E4BP4.01	2266	2286	2276	1.000	atgatgttacGTAAtcgctga
174	V$CREL.01	1225	1237	1231	1.000	attggcatTTCCc
175	V$VAX1.01	2195	2213	2204	1.000	acacattAATTatcttgct
176	V$SPZ1.01	3783	3793	3788	1.000	aGGAGggtccc
177	V$NKX61.01	3802	3816	3809	1.000	agagTTAAttagccc
178	V$STAT3.02	1823	1841	1832	1.000	cagaTTCCtggattagga
179	V$AARE.01	801	809	805	0.885	tTTCCatca
180	V$ISRE.01	1245	1265	1255	1.000	ctgaaaaaaGAAActgcttc
181	V$HIC1.01	662	674	668	1.000	tggggTGCCcaga
182	V$SATB1.01	636	650	643	1.000	aatAATAaaaaacac
183	V$IRF1.01	3063	3083	3073	1.000	cctagaaaatGAAAgcctttc
184	V$STAT.01	2821	2839	2830	1.000	ctccttaggGGAAaatcca
185	V$LMX1B.01	2189	2211	2200	1.000	cataacagcaagaTAATtaatgt
186	V$OCT1.03	2196	2212	2204	1.000	gcaagataATTAatgtg
187	V$RBPJK.02	1195	1207	1201	1.000	gtgaTGGGaaaat
188	V$NFAT.01	800	818	809	1.000	ctgatGGAAAgaagatctt
189	V$NKX61.01	2198	2212	2205	1.000	cacaTTAAttatctt
190	V$SIX4.01	2137	2151	2144	1.000	atcaagGTGTcagcg
191	V$NBRE.01	883	897	890	1.000	aaagAAGGtcatgga
192	V$SIX.01	2956	2968	2962	1.000	aacTCAGgtatct
193	V$VBP.01	1846	1862	1854	1.000	ctgtataatGTAAccta
194	V$ZF5.01	2318	2328	2323	1.000	ctggGCGCctg
195	V$GSH2.01	2662	2680	2671	1.000	cgtataTAATtagggctta
196	V$AHRARNT.03	3170	3194	3182	1.000	cgaggggaatGCGTggcttcggtc
197	V$HNF4A.01	773	797	785	1.000	taattcactcCAAAgtacagggtta
198	V$ZF5.01	3632	3642	3637	1.000	ctgaGCGCctg
199	V$NANOG.01	2200	2218	2209	1.000	gataattAATGtgtgctgt
200	V$HHEX.01	1540	1558	1549	1.000	ttttctttttTAATgttg
201	V$LHX6.01	3798	3820	3809	1.000	gctaagagtTAATtagccccgca
202	V$NANOG.01	270	288	279	1.000	cttcatgAATGtttggaga
203	V$BRN2.03	2196	2214	2205	0.929	cacacattaATTAtcttgc

204	V$MTBF.01	1039	1047	1043	1.000	tggaATTTg
205	V$GATA1.03	2196	2208	2202	1.000	gcaaGATAattaa
206	V$TH1E47.01	1662	1682	1672	1.000	agagcccttCCAGactcctgt
207	O$PTATA.02	1121	1135	1128	1.000	tggtTATAaatatgc
208	V$ZF5.01	3260	3270	3265	1.000	gggcGCGCacg
209	V$GSH2.01	2663	2681	2672	1.000	aagcccTAATtatatacgt
210	V$WHN.01	3430	3440	3435	1.000	gcgACGCcgcg
211	V$GSH2.01	2234	2252	2243	1.000	tcgtggTAATttgttatag
212	V$LMX1B.01	612	634	623	1.000	ctctgtctcaaaaTAATaaaaaa
213	V$HSF2.02	300	324	312	1.000	ggggaagtaaaaAGAAgggtccaaa
214	V$CEBPB.01	2086	2100	2093	1.000	aaggtgagGAAAtcg
215	V$IRF4.01	1534	1554	1544	1.000	attaaaaaaaGAAAaaagaaa
216	V$CDPCR3HD.01	2088	2106	2097	0.886	gaaaatcGATTtcctcacc
217	V$RBPJK.02	1230	1242	1236	1.000	agttTGGGaaatg
218	V$BRN2.04	3800	3818	3809	1.000	taagagtTAATtagccccg
219	O$DINR.01	2572	2582	2577	1.000	gaTCAGtcggt
220	V$PTX1.01	2461	2477	2469	1.000	agggCTAAcccctctaa
221	V$BRIGHT.01	2196	2214	2205	1.000	gcaagataATTAatgtgtg
222	V$NFAT.01	3220	3238	3229	1.000	aatttGGAAataagctgga
223	V$P53.03	2896	2918	2907	0.922	ctctggagcagggggCAAGtcag
224	V$PEA3.01	3437	3457	3447	1.000	ggaaagaAGGAagggctgcga
225	V$IRF3.01	3073	3093	3083	1.000	cggggaaatgGAAAggctttc
226	V$SOX2.01	2734	2756	2745	1.000	aaataaaACAAagacccatctct
227	V$TWIST.01	665	685	675	0.952	aagaataCATGtggggtgccc
228	V$CDP.02	1216	1234	1225	1.000	aaatgcCAATaaccatagc
229	V$SMAD4.01	837	845	841	1.000	tGTCTtgcc
230	V$SREBP.03	391	405	398	1.000	agaTCACctgagcgc
231	V$HLF.01	2809	2825	2817	1.000	atccattccGTAAccga
232	V$AREB6.01	2957	2969	2963	1.000	aagatACCTgagt
233	V$CEBPB.01	3226	3240	3233	1.000	gcaatttgGAAAtaa
234	V$YY1.02	276	296	286	1.000	cccaaCCATctccaaacattc
235	O$VTATA.01	1156	1172	1164	1.000	aggtaTAAAtttcagga
236	V$E47.02	348	364	356	1.000	ggattacaGGTGtaagc
237	V$CDP.02	1604	1622	1613	1.000	tcccacCAATctcttccct
238	V$OCT1.03	2197	2213	2205	1.000	acacattaATTAtcttg
239	V$NANOG.01	3170	3188	3179	1.000	cgaggggAATGcgtgggct
240	V$CDP.02	2749	2767	2758	1.000	atggatCAATgaaataaaa
241	O$VTATA.01	1118	1134	1126	1.000	ggttaTAAAtatgctca
242	V$NANOG.01	2812	2830	2821	1.000	gttacggAATGgattttcc
243	V$MYT1L.01	3891	3903	3897	1.000	ggagAGTTggatg
244	V$HOXA3.01	2664	2682	2673	1.000	agcccTAATtatatacgta
245	V$SIX3.02	352	372	362	1.000	acacctgTAATcccagcactt
246	V$RBPJK.02	2967	2979	2973	1.000	ggaaTGGGaaaag
247	V$TEAD.01	2251	2263	2257	1.000	tccCATTcctgct
248	V$HOXC8.01	2661	2679	2670	1.000	gtatataATTAgggcttat
249	V$NEUROG.01	3657	3669	3663	1.000	ttgCCATctgata
250	V$NFKAPPAB.01	3300	3312	3306	1.000	gcGGGAgatcccg
251	V$HOXD10.01	620	636	628	1.000	caaaataaTAAAaaata
252	V$CRX.01	494	510	502	1.000	atctgTAATcccagcta
253	V$IRX5.01	670	682	676	1.000	aataCATGtgggg
254	V$LHX6.01	3799	3821	3810	1.000	gtgcggggcTAATtaactcttag
255	V$SP1.03	3513	3527	3520	1.000	agaGGGCgggagcag

256	V$LEF1.02	1644	1660	1652	1.000	cacatctCAAAgccggt
257	O$DINR.01	3066	3076	3071	0.969	ttTCATttct
258	V$NFKAPPAB.01	3301	3313	3307	1.000	ccGGGAtctcccg
259	V$OCT1.03	2664	2680	2672	1.000	cgtatataATTAgggct
260	V$IPF1.01	2198	2216	2207	1.000	agcacacatTAATtatctt
261	V$TST1.01	3217	3235	3226	0.900	ttggaAATAagctggaccc
262	V$ZBP89.01	1338	1360	1349	1.000	cacacacactCCCCctaggcctg
263	V$LHX6.01	2193	2215	2204	1.000	acagcaagaTAATtaatgtgtgc
264	V$OCT1.03	3801	3817	3809	1.000	aagagttaATTAgcccc
265	V$IRX5.01	671	683	677	1.000	cccaCATGtattc
266	V$HIC1.01	3712	3724	3718	1.000	taggcTGCCggcg
267	V$RFX1.01	1474	1492	1483	1.000	agagatacatgGCAActtg
268	V$IRF7.01	2252	2272	2262	0.937	gcagGAATgggaaaatgatgt
269	V$CREL.01	3346	3358	3352	1.000	gccgggcTTCCc
270	V$LMX1B.01	625	647	636	1.000	taataaaaaataaTAATaaaaaa
271	V$MEF3.01	126	138	132	1.000	tggTCAGgagtcc
272	V$MYF5.01	3390	3406	3398	1.000	ctgcggCAGCcgctggg
273	V$USF.04	2762	2774	2768	0.915	taggCACAtggat
274	V$NKX12.01	3801	3817	3809	1.000	ggggctAATTaactctt
275	V$INSM1.01	434	446	440	1.000	tgataGGGGgaag
276	V$HOXD10.01	633	649	641	1.000	aataataaTAAAaaaca
277	V$SCX.01	2758	2778	2768	1.000	attgatccatgTGCCtataac
278	V$XFD1.01	2414	2430	2422	1.000	attttgTAAAtaaggtc
279	V$HIC1.01	3275	3287	3281	0.869	aaggcTGCGcgaa
280	V$GATA.01	1597	1609	1603	1.000	gtggGATAaggaa
281	V$HIC1.01	1680	1692	1686	0.889	acgggTGCAcaca
282	V$ESRRB.01	880	898	889	1.000	ggaaaagaAGGTcatggag
283	V$MARE.02	964	984	974	0.875	gcaGGCTaatgcagcagcggc
284	V$LHX3.02	3794	3816	3805	1.000	gccggctaagagtTAATTagccc
285	V$ER.04	1395	1413	1404	1.000	ctagGTCAtgcagacccta
286	V$OCT3_4.02	1394	1410	1402	1.000	ggtctGCATgacctaga
287	V$INSM1.01	25	37	31	1.000	tggaaGGGGacac
288	V$BRN2.03	679	697	688	1.000	tattcttcATTTaaatat
289	V$WT1.01	3821	3837	3829	0.837	cggcgAGGGgggaggcg
290	V$GATA3.01	1510	1522	1516	1.000	tcggGATAaaaat
291	V$E47.01	3412	3428	3420	1.000	ggcttGCAGgtggttcg
292	V$NFY.04	1219	1233	1226	1.000	aatgCCAAtaaccat
293	V$HMX3.01	2163	2181	2172	1.000	gaagtccaAAGTggtctc
294	V$MYT1L.01	3244	3256	3250	1.000	ggacAGTTcacgc
295	V$GFI1.02	2141	2155	2148	1.000	caaAATCaaggtgtc
296	V$SRY.01	1055	1077	1066	1.000	tggtaaaACAAaaaaatttttg
297	V$MIT.01	2760	2774	2767	1.000	tgatcCATGtgccta
298	V$HOXD10.01	2745	2761	2753	1.000	caatgaaaTAAAacaaa
299	V$NMYC.01	3239	3251	3245	1.000	gccccgCGTGaac
300	V$CETS1P54.01	703	723	713	0.843	ctcttcCTGGaagtaaatatt
301	V$DLX3.01	1425	1443	1434	1.000	ggcttcagcTAATttcatc
302	V$BRN4.01	2667	2685	2676	1.000	gtatacgtataTAATtagg
303	V$HMGA.01	765	787	776	1.000	ctttggagtgAATTattggagtt
304	V$NFKAPPAB.01	3344	3356	3350	1.000	cgGGGAagcccg
305	V$OCT1.03	3802	3818	3810	1.000	cggggctaATTAactct
306	V$ZNF219.01	1341	1363	1352	1.000	acacactCCCCctaggcctgcag
307	V$PCE1.01	3801	3817	3809	1.000	ggggcTAATtaactctt

308	V$VMYB.05	1684	1696	1690	1.000	gggAACGggtgca
309	V$IPF1.01	2665	2683	2674	1.000	atacgtataTAATtagggc
310	V$NFKAPPAB50.01	438	450	444	1.000	aggGGGAagccct
311	V$AP2.02	1503	1517	1510	1.000	aaaGCCTtcggata
312	V$HMGIY.01	1157	1179	1168	1.000	ggtataAATTtcaggatatatgt
313	V$PAX4.02	2664	2678	2671	1.000	tatatAATTagggct
314	V$HOXC13.02	1109	1125	1117	1.000	tatgctcaTAAAgaagt
315	V$TST1.01	167	185	176	0.900	attgtTTTAagctgctaaa
316	V$MYOD.01	2559	2575	2567	0.931	cggtGACAgatggagac
317	V$P53.02	1804	1826	1815	1.000	ctctcctcccatgttCATGtcct
318	V$FAST1.02	157	173	165	1.000	aaataTGTGcattgttt
319	V$BRIGHT.01	3800	3818	3809	1.000	cggggctaATTAactctta
320	V$NF1.03	1467	1487	1477	1.000	tctctgccaagttGCCAtgta
321	V$BACH2.01	2001	2021	2011	0.813	caatcgTGGGtcaagttccta
322	V$HES1.01	3260	3274	3267	0.944	agggcgtGCGCgccc
323	V$HES1.01	3356	3370	3363	0.944	tgggcgtGCGCagcc
324	V$GATA1.04	791	803	797	1.000	tcagGATAaccct
325	V$IPF1.01	2193	2211	2202	1.000	acagcaagaTAATtaatgt
326	V$NF1.03	1625	1645	1635	1.000	agagtacctccctGCCAcaca
327	V$BRN2.03	2744	2762	2753	1.000	ctttgttttATTTcattga
328	V$AP2.02	1346	1360	1353	1.000	cagGCCTagggggag
329	V$KAISO.01	2847	2857	2852	1.000	ttgcCTGCgaa
330	V$HMGIY.01	1046	1068	1057	1.000	aaaaaaAATTtttgagaggggtg
331	V$LHX6.01	2194	2216	2205	1.000	agcacacatTAATtatcttgctg
332	V$GSC.02	109	125	117	1.000	ccagaTAATccatgcct
333	V$ZNF219.01	1731	1753	1742	1.000	cagtcaaCCCCcagccccctggt
334	V$OCT1.03	2663	2679	2671	1.000	aagcccctaATTAtatac
335	V$LHX6.01	2660	2682	2671	1.000	aataagcccTAATtatatacgta
336	V$P53.02	1783	1805	1794	1.000	gatctcaggcggaatCATGccct
337	V$CDX2.02	1119	1137	1128	1.000	gagcatatTTATaaccata
338	V$ZNF219.01	2897	2919	2908	1.000	tgacttgCCCCctgctccagagc
339	V$ZNF217.01	766	778	772	1.000	GAATtattggagt
340	V$BRN5.04	3799	3821	3810	1.000	gtgcggggctaATTAactcttag
341	V$BRN2.04	2663	2681	2672	1.000	acgtataTAATtagggctt
342	V$GATA3.01	1894	1906	1900	1.000	ggagGATAataaa
343	V$MAZ.01	3481	3493	3487	1.000	gggaGAGGggcgc
344	V$HOXC13.01	1894	1910	1902	1.000	ggaggataaTAAActga
345	V$GRHL1.01	3565	3577	3571	1.000	agagccGGTTccc
346	V$MYCMAX.03	2044	2056	2050	0.754	cggccaTGAGcca
347	V$CAAT.01	11	25	18	0.847	ataaCCAGtgaactt
348	V$INSM1.01	3843	3855	3849	1.000	tttctGGGGacac
349	V$CHREBP_MLX.01	2629	2645	2637	1.000	CACGtggacccgggtg
350	V$TIEG.01	1339	1353	1346	1.000	agGGGGagtgtgtgt
351	V$P53.02	2885	2907	2896	0.885	cagcccgagctctgaCTTGcccc
352	V$RFX1.02	1992	2010	2001	0.882	aagatgcaataGGAActtg
353	V$INSM1.01	1044	1056	1050	1.000	tgagaGGGGtgga
354	V$AP2.01	3788	3802	3795	0.881	ttaGCCGgcgggacc
355	V$E4F.01	848	860	854	0.842	gtgATGTgacaca
356	V$SP1.03	1734	1748	1741	1.000	aggGGGCtgggggtt
357	V$CDE.01	3748	3760	3754	1.000	gcagCGCGgcatc
358	V$PRE.01	1024	1042	1033	1.000	tttggagaagaTGTTctca
359	V$GC.01	2915	2929	2922	0.877	atagGGAGggctct

360	O$HMTE.01	2421	2441	2431	1.000	tgAGCGgggcgattttgtaaa
361	V$INSM1.01	289	301	295	1.000	tggttGGGGcagg
362	V$GATA3.02	808	820	814	1.000	gaaAGATcttctt
363	V$EN1.01	684	702	693	1.000	ttgggataTTTAaatgaaa
364	V$HOX_PBX.01	2353	2369	2361	0.944	acatGGATggattgttc
365	V$DLX5.01	2236	2254	2245	1.000	tgctataacaAATTaccac
366	V$MAZ.01	3167	3179	3173	1.000	aaccGAGGggaat
367	V$TLX1.01	1981	1999	1990	1.000	attaaagCGGTaagatgca
368	V$BACH2.01	2373	2393	2383	0.813	tgctgaTGTGtcagagacgag
369	V$MEF2.03	1552	1574	1563	1.000	aatgttgctcaaaATAGttttct
370	V$CP2.01	1421	1439	1430	1.000	tgCTGGcttcagctaattt
371	V$LHX3.02	2665	2687	2676	1.000	ctgtatacgtataTAATtagggc
372	V$E2F.03	3257	3273	3265	1.000	cgcggGCGCgcacgccc
373	V$TEAD.01	3171	3183	3177	1.000	acgCATTccctc
374	V$NFKAPPAB.01	1227	1239	1233	1.000	ttGGGAaatgcca
375	V$OLF1.02	2971	2993	2982	1.000	tcccatTCCCtcgggttgtgag
376	V$PRE.01	2354	2372	2363	1.000	catggatggatTGTTcttc
377	V$OCT3_4.02	2782	2798	2790	1.000	tttgtGCATctggccga
378	V$PAX4.02	2197	2211	2204	1.000	acattAATTatcttg
379	V$AML3.01	2685	2699	2692	1.000	cagaGTGGttttat
380	V$GFI1.02	1412	1426	1419	1.000	tacAATCaatgctgg
381	V$NKX25.02	2664	2682	2673	1.000	agccTAATtatatacgta
382	V$HMBOX.01	7	23	15	1.000	gttcactgGTTAttgat
383	V$PAX4.02	2665	2679	2672	1.000	gccctAATTatatac
384	V$TST1.01	970	988	979	1.000	gctgcATTAgcctgctgct
385	V$TEAD.01	271	283	277	1.000	aaaCATTcatgaa
386	V$HMGA.01	267	289	278	1.000	aatcttcatgAATGtttggagat
387	V$NKX12.01	3802	3818	3810	1.000	agagttAATTagccccg
388	O$VTATA.01	2665	2681	2673	0.892	acgtaTATAattagggc
389	V$MAZR.01	2916	2928	2922	1.000	taggaGGGGctc
390	V$TAL1BETAITF2.01	3653	3673	3663	1.000	agggtatCAGAtggcaaagtt
391	V$TEF.01	2418	2434	2426	1.000	ggcgattttGTAAataa
392	V$IPF1.01	2660	2678	2669	1.000	aataagcccTAATtatata
393	V$CDX2.02	148	166	157	1.000	gcacatatTTATtatctca
394	V$PLAG1.01	1614	1634	1624	1.000	GAGGtactctacagggaagag
395	V$MTF-1.01	3356	3370	3363	0.949	ggctGCGCacgccca
396	V$FHXB.01	1556	1572	1564	0.818	ttgctcAAAAtagtttt
397	V$HSF1.01	263	287	275	0.857	ttggaatcttcaTGAAtgtttggag
398	V$TST1.01	1976	1994	1985	1.000	ggagcATTAaagcggtaag
399	V$CAAT.01	1078	1092	1085	1.000	ctagCCAAgcacttt
400	V$BRN2.04	2195	2213	2204	1.000	agcaagaTAATtaatgtgt
401	V$BRN5.04	2660	2682	2671	1.000	aataagcctaATTAtatacgta
402	V$CDX2.02	1895	1913	1904	1.000	aagtcagtTTATtatcctc
403	V$MYOGENIN.02	3006	3022	3014	1.000	gagatcCAGCtgcgaca
404	V$RFX1.02	197	215	206	0.945	ttattacacagGTAActaa
405	V$OCT3_4.02	160	176	168	1.000	tatgtGCATtgttttaa
406	O$PTATA.02	1155	1169	1162	1.000	gaggTATAaatttca
407	V$BRN3.02	766	784	775	1.000	actccaaTAATtcactcca
408	V$NKX12.01	2663	2679	2671	1.000	gtatatAATTagggctt
409	V$PLAG1.01	3506	3526	3516	1.000	GAGGgcgggagcaggccagg
410	V$BRN2.01	2668	2686	2677	0.933	ctAATTatatacgtataca
411	V$CHOP.01	1587	1599	1593	1.000	ttttGCAAtattc

412	V$XFD2.01	1119	1135	1127	1.000	tggttaTAAAtatgctc
413	V$TST1.01	2230	2248	2239	1.000	aacaaATTAccacgaatgt
414	V$MTBF.01	2961	2969	2965	0.885	aggtATCTt
415	V$TAL1ALPHAE47.01	2556	2576	2566	1.000	tcggtgaCAGAtggagacccg
416	V$NFKAPPAB.01	3783	3795	3789	1.000	gcGGGAccctcct
417	V$TAL1BETAE47.01	465	485	475	1.000	ctgcgccCAGAtgattttgta
418	V$BCL6.02	3058	3074	3066	1.000	catatccTAGAaaatga
419	V$MEF3.01	3149	3161	3155	1.000	ggcTCAGggcact
420	V$PAX4.02	2198	2212	2205	1.000	aagatAATTaatgtg
421	V$ZNF217.01	1869	1881	1875	1.000	GAATccaggcagt
422	O$VTATA.01	2687	2703	2695	1.000	gaacaTAAAaaccactc
423	V$MTBF.01	2795	2803	2799	1.000	agttATTTg
424	V$INSM1.01	871	883	877	1.000	ttcctGGGGcttg
425	V$GABP.01	1660	1680	1670	1.000	aggagtctGGAAggctctga
426	V$MSX3.01	2233	2251	2242	1.000	ttcgtggTAATttgttata
427	V$MEIS1B_HOXA9.01	2140	2154	2147	1.000	TGACaccttgatttt
428	V$NXF_ARNT.01	3679	3703	3691	1.000	cttcataaaggCGTGgtggcgattc
429	V$PHOX2.01	2511	2531	2521	1.000	acctcTAATgtcctggtcccg
430	V$NKX25.02	3802	3820	3811	1.000	agagtTAATtagccccgca
431	V$OVOL1.01	2715	2729	2722	1.000	aagccGTTAaatag
432	V$HOMEZ.01	2092	2106	2099	1.000	aggaaATCGattttc
433	V$LHX6.01	2661	2683	2672	1.000	atacgtataTAATtagggcttat
434	V$ATF2.01	3718	3738	3728	0.815	cctgtcTGATgtcataggctg
435	V$DLX4.01	764	782	773	1.000	gaactccaatAATTcactc
436	V$REV-ERBA.01	1649	1671	1660	1.000	ctcaagccgGTCAgagcccttc
437	V$XFD2.01	150	166	158	1.000	agataaTAAAtatgtgc
438	V$HSF1.01	262	286	274	0.857	tccaaacattcaTGAAgattccaag
439	V$JARID2.01	3058	3076	3067	0.850	tttcaTTTTctaggatatg
440	O$HMTE.01	3403	3423	3413	0.961	gcAGCCccgcgaaccacctgc
441	V$CKROX.01	3826	3842	3834	1.000	agggGGGAggcgccagt
442	V$HNF1.04	204	220	212	1.000	tgctgttaGTTAcctgt
443	V$PHOX2.01	1877	1897	1887	1.000	ctcccTAATctcatccactgc
444	V$MTF-1.01	3511	3525	3518	0.835	ccctGCTCccgccct
445	V$LEF1.01	1856	1872	1864	1.000	tatacagCAAAgggaat
446	V$E4F.01	2268	2280	2274	1.000	attACGTaacatc
447	V$LEF1.01	212	228	220	1.000	ctaacagCAAAggagag
448	V$PAX6.04	1603	1621	1612	1.000	atcCCACcaatctcttccc
449	V$JARID2.01	1056	1074	1065	0.850	aaaaaTTTTttttgtttta
450	O$VTATA.02	727	743	735	1.000	atctgTAAAatggagtt
451	V$IRF7.01	2962	2982	2972	0.937	gaggGAATgggaaaagatacc
452	V$OLF1.02	2336	2358	2347	1.000	ccatgtTCCCtttggggatctag
453	V$CLOX.01	112	130	121	0.804	gataATCCatgcctggact
454	V$SL1.01	2919	2941	2930	1.000	ccccctccCTATttagagcccagt
455	V$CDX2.02	2743	2761	2752	1.000	tctttgttTTATttcattg
456	V$IRF7.01	224	244	234	1.000	gagaGAAAgccaaaatgaagg
457	V$EGR2.01	3176	3192	3184	1.000	gaatGCGTgggcttcgg
458	V$PU1.01	2085	2105	2095	1.000	aaaggtgaGGAAatcgatttt
459	V$NFAT5.02	1198	1216	1207	1.000	atgGGAAaattgaggggg
460	V$NFKAPPAB.01	2821	2833	2827	1.000	agGGGAaaatcca
461	V$HOXB9.01	3642	3658	3650	1.000	gtcagtagTAAAgggta
462	V$SP1.01	292	306	299	1.000	ttgGGGCaggggaag
463	V$BARBIE.01	980	994	987	1.000	tcgaAAAGcagcagg

464	V$GLI3.02	3175	3189	3182	1.000	aagcCCACgcattcc
465	V$OLF1.02	1341	1363	1352	1.000	acacacTCCCcctaggcctgcag
466	V$PHOX2.01	2470	2490	2480	1.000	ccctcTAATgtgttttcagca
467	V$PLZF.01	785	799	792	1.000	aagTACAgggttatc
468	V$E2F.03	985	1001	993	0.810	gctagGCTCgaaaagca
469	V$PLAG1.01	3514	3534	3524	1.000	GAGGcacagagggcggagca
470	V$PLZF.01	3858	3872	3865	1.000	gagTACAgtggcagc
471	V$GLIS3.01	1206	1220	1213	1.000	atagCCCCcctcaat
472	V$HOMEZ.01	2093	2107	2100	1.000	ggaaaATCGatttcc
473	V$OLF1.02	2400	2422	2411	1.000	tgtctaTCCCtgaagaccttatt
474	V$GC.01	3827	3841	3834	0.877	ggggGGAGgcgccag
475	V$E4F.01	2266	2278	2272	0.842	atgATGTtacgta
476	V$VDR_RXR.04	3234	3258	3246	0.929	cgggacaGTTCacgcgggcaattt
477	V$LEF1.01	2739	2755	2747	1.000	aataaaaCAAAgaccca
478	V$RREB1.01	1693	1707	1700	1.000	tCCCAaacctccctc
479	V$CLOX.01	2093	2111	2102	1.000	ggaaATCGattttccccta
480	V$BARBIE.01	1703	1717	1710	1.000	aaggAAAGtggaggg
481	V$HSF2.01	253	277	265	0.917	acagaattgcttgGAATcttcatga
482	V$PPARG.02	2493	2515	2504	0.892	aggggatgggcAGAGgccggga
483	V$PPARG.02	3315	3337	3326	1.000	cttctgccggcgAAAGggaagcg
484	V$STAT1.02	1825	1843	1834	1.000	ctaatcccaGGAAtctgtg
485	V$EVI1.02	3200	3216	3208	1.000	ctgagAAGAaaagcttc
486	O$LTATA.01	2663	2679	2671	1.000	gtaTATAattagggctt
487	V$E4BP4.01	1843	1863	1853	1.000	gctgtataatGTAAcctattc
488	V$VMYB.01	211	223	217	0.817	actAACAgcaaag
489	V$DLX4.01	1454	1472	1463	1.000	ctgaaaaaatAATTctctg
490	V$BNC.01	247	265	256	1.000	caagcaattcTGTCccttt
491	V$PIT1.02	2199	2213	2206	1.000	acacaTTAAttatct
492	V$RBPJK.01	2867	2879	2873	1.000	agctTGGGaaggc
493	V$CTCF.04	3285	3311	3298	0.841	gggatctcccgccagcCGGCtgcaaag
494	V$BRN2.02	675	693	684	1.000	catgtattcttTCATttaa
495	V$EVI1.05	2958	2974	2966	1.000	ggaaaaGATAcctgag
496	V$GLI1.01	420	434	427	0.771	aagaccaGCCAagct
497	V$HNF6.01	2093	2109	2101	0.786	ggggaaaaTCGAtttcc
498	V$PPARG.02	235	257	246	1.000	aaaatgaaggggAAAGgacaga
499	V$MTF-1.01	1681	1695	1688	1.000	gtgtGCACccgttcc
500	V$NKX12.01	2197	2213	2205	1.000	caagatAATTaatgtgt
501	V$MYT1.02	3666	3678	3672	1.000	gcaAAGTtgggac
502	V$HSF2.01	2861	2885	2873	1.000	gggcttagcttggGAAGgctcaaaa
503	V$MARE.02	3274	3294	3284	0.792	ggcTGCAaaggctgcgcgaaa
504	O$PTATA.01	685	699	692	0.826	ttcaTTTAaatatcc
505	V$ZBRK1.01	1246	1270	1258	1.000	gcagggaaGCAGtttctttttttca
506	V$HOXA10.01	148	164	156	1.000	tgagataaTAAAtatgt
507	V$SZF1.01	3651	3675	3663	1.000	aaaGGGTatcagatggcaaagttgg
508	V$PHOX2.01	768	788	778	1.000	tccaaTAATtcactccaaagt
509	V$NFAT5.01	3218	3236	3227	1.000	tttGGAAAataagctggacc
510	V$VMYB.04	2124	2136	2130	1.000	actAACGcagccc
511	V$HOXB9.01	2579	2595	2587	1.000	cgagtcctTAAAagatc
512	V$HOXB9.01	3676	3692	3684	1.000	gaccttcaTAAAggcgt
513	V$PLAG1.01	3171	3191	3181	1.000	GAGGggaatgcgtgggcttcg
514	V$PLAG1.01	2902	2922	2912	0.958	GGGGctctggagcaggggca
515	V$TCFCP2L1.01	940	958	949	0.815	gcCAGGctctgtcctgcaa

516	O$HMTE.01	3423	3443	3433	0.961	caAGCCccgcggcgtcgcagc
517	V$TIEG.01	1337	1351	1344	0.750	ggGGAGtgtgtgtgt
518	**V$GKLF.01**	**306**	**322**	**314**	**1.000**	**gtaaaaagaAGGGtcca**
519	V$SL1.01	1553	1575	1564	1.000	gagaaaaCTATttgagcaacat
520	V$BRN5.04	188	210	199	1.000	tctagcaacttATTAcacaggta
521	V$TIEG.01	1285	1299	1292	0.750	gtGTGGtgtgtgtgt
522	V$GRE.01	2688	2706	2697	1.000	agtggtttttatGTTCtga
523	V$TIEG.01	1283	1297	1290	0.750	gtGGTGtgtgtgtgt
524	V$STAT.01	1925	1943	1934	0.769	aaccttacgGCAAaaataa
525	V$ESRRB.01	1389	1407	1398	1.000	gctcatctAGGTcatgcag
526	V$BRN2.01	196	214	205	0.967	ctTATTacacaggtaacta
527	V$HEN1.02	3004	3024	3014	1.000	tgtgtcgcaGCTGgatctcca
528	O$XCPE1.01	3787	3797	3792	1.000	cgGCGGgaccc
529	V$GRHL1.01	10	22	16	1.000	ttcactGGTTatt
530	V$NKX12.01	2196	2212	2204	1.000	cacattAATTatcttgc
531	V$SOX5.01	2535	2557	2546	1.000	cgaggtgaCAATagctcatgggg
532	V$HNF6.01	2090	2106	2098	0.786	tgaggaaaTCGAtttc
533	V$ACAAT.01	2012	2026	2019	0.750	gccaGCAAtcgtggg
534	V$EVI1.06	2193	2209	2201	1.000	acagcaAGATaattaat
535	V$NKX63.01	2665	2679	2672	1.000	gtataTAATtagggc
536	V$NKX63.01	2197	2211	2204	1.000	caagaTAATtaatgt
537	V$PAX6.02	3008	3026	3017	1.000	tctggagatCCAGctgcga
538	V$SL1.01	2661	2683	2672	0.803	ataagccCTAAttatatacgtat
539	V$OCT2.01	1219	1235	1227	1.000	gaaATGCcaataaccat
540	V$RBPJK.01	815	827	821	0.796	agtgTGAGaaaga
541	V$PHOX2.01	963	983	973	1.000	caggcTAATgcagcagcggca
542	V$GLI1.01	3383	3397	3390	1.000	cggagctCCCAgcgg
543	V$GSH1.01	1574	1592	1583	1.000	tctgctcagaTAATtttgc
544	V$HBP1.01	2748	2770	2759	1.000	cacatggatcAATGaataaaac
545	V$BRN2.04	3801	3819	3810	1.000	gcggggcTAATtaactctt
546	V$ARP1.01	3495	3519	3507	1.000	ggagcaggccaggGGTCgggaatc
547	V$HBP1.01	2965	2987	2976	1.000	accccgagggAATGggaaaagat
548	V$EN1.02	2233	2251	2242	1.000	tataacaAATTaccacgaa
549	V$CDE.01	3025	3037	3031	0.842	gatgCGCCgtgtc
550	V$HFH1.01	1917	1933	1925	1.000	caaaaaTAAActctatc
551	V$HOXC9.01	2197	2213	2205	1.000	caagataaTTAAtgtgt
552	V$SRF.04	1144	1162	1153	1.000	tatacctccTATGgcttgg
553	V$HOXC9.01	3801	3817	3809	1.000	ggggctaaTTAActctt
554	V$HBP1.01	2809	2831	2820	1.000	tcggttacggAATGgattttccc
555	V$CDE.01	3475	3487	3481	1.000	ggggCGCGagcta
556	V$PPARG.03	2073	2095	2084	1.000	ctagaagctggaAAAGgtgagga
557	V$SREBP.02	664	678	671	0.750	tggGCACcccacatg
558	V$DBP.01	2182	2198	2190	1.000	tgctgTTATggagggca
559	V$VDR_RXR.04	1382	1406	1394	0.821	ctggccaGCTCatctaggtcatgca
560	V$NRF2.01	3191	3211	3201	1.000	ggtccgtcGGAAgcttttctt
561	V$SL1.01	442	464	453	1.000	ggaagccCTATctctactaaaaa
562	V$HOXC9.01	1121	1137	1129	0.884	tatggttaTAAAtatgc
563	V$TEF.01	3721	3737	3729	0.818	ctgtctgatGTCAtagg
564	V$NFY.03	1520	1534	1527	1.000	aaagCCAAacagatt
565	V$HDBP1_2.01	3319	3337	3328	1.000	cttctgCCGGcgaaaggga
566	**V$GKLF.01**	**2915**	**2931**	**2923**	**0.826**	**aaatagggaGGGGctct**
567	V$BRN2.02	151	169	160	0.857	aatgcacatatTTATtatc

568	V$GKLF.01	234	250	242	0.826	caaaatgaaGGGGaaag
569	V$NBRE.01	2082	2096	2089	1.000	ggaaAAGGtgaggaa
570	V$NFKAPPAB50.01	3460	3472	3466	0.750	gagCGGAtgcccg
571	V$CLOX.01	2758	2776	2767	0.804	attgATCCatgtgcctata
572	V$DBP.01	1395	1411	1403	0.808	ctaggTCATgcagaccc
573	V$BRN3.03	2199	2217	2208	1.000	agataatTAATgtgtgctg
574	V$ER.03	1395	1413	1404	1.000	tagggtctgcatGACCtag
575	V$TCF2.01	2710	2726	2718	0.756	accaactaTTTAacggg
576	V$RP58.01	469	481	475	1.000	aaatCATCtgggc
577	V$ISL1.01	1891	1913	1902	1.000	tagggaggaTAATaaactgactt
578	V$EN2.01	767	785	776	1.000	ctccaatAATTcactccaa
579	V$TCF2.01	3802	3818	3810	0.761	cggggctaATTAactct
580	V$AIRE.01	1078	1104	1091	0.964	aaagtgcttggctagagAGGAgaaatg
581	V$IRF7.01	236	256	246	0.821	aaatGAAGgggaaagggacag
582	V$OCT1.03	633	649	641	1.000	tgtttttATTAttatt
583	V$FHXB.01	654	670	662	0.818	gtgcccAGAAcaatttt
584	V$ISL1.01	1971	1993	1982	1.000	ttaccgcttTAATgctcccttcc
585	V$HNF6.01	2816	2832	2824	0.833	ggggaaaaTCCAttccg
586	V$AIRE.01	692	718	705	0.917	ttacttccaggaagagtTGGGatattt
587	V$RP58.01	3020	3032	3026	1.000	ggcgCATCtggag
588	V$HNF1.04	3797	3813	3805	1.000	ggctaagaGTTAattag
589	V$OCT1.03	620	636	628	1.000	tatttttATTAttttg
590	V$GSH1.01	2199	2217	2208	1.000	cagcacacatTAATtatct
591	V$GSH1.01	970	988	979	1.000	agcagcaggcTAATgcagc
592	V$HOX1-3.01	965	983	974	1.000	caggcTAATgcagcagcgg
593	V$PIT1.02	3801	3815	3808	1.000	aagagTTAAttagcc
594	V$HOXB5.01	1892	1910	1901	0.763	tcagtTTATtatcctccct
595	V$HDBP1_2.01	3786	3804	3795	1.000	tcttagCCGGcgggaccct
596	V$NKX26.01	2161	2179	2170	1.000	agtccaaAGTGggtctcag
597	V$HOXC10.01	1907	1923	1915	1.000	ctctatcaTAAAgtcag
598	V$OVOL1.01	2121	2135	2128	1.000	ggctgcGTTAgtttc
599	V$IK3.01	1138	1150	1144	1.000	ggcttGGAAtatc
600	V$HOXA9.02	2690	2706	2698	1.000	tcagaacaTAAAaacca
601	V$OCT1.06	1216	1232	1224	0.750	gctatggtTATTggcat
602	V$HNF6.01	722	738	730	0.833	tttctaacTCCAtttta
603	V$HOX1-3.01	1938	1956	1947	1.000	aaggtTAATgtagaagagg
604	V$AML3.01	1287	1301	1294	1.000	gagtGTGGtgtgtgt
605	V$E2F.03	1312	1328	1320	1.000	cacacGCGCgcacacac
606	V$OLF1.01	3868	3890	3879	1.000	gtgagcTCCCctgggtaggagta
607	V$BRN2.04	2662	2680	2671	1.000	taagcccTAATtatatacg
608	V$PIT1.02	2200	2214	2207	1.000	gataaTTAAtgtgtg
609	V$GCM1.01	3159	3169	3164	1.000	gcCCCTcaaac
610	V$NKX63.01	2201	2215	2208	1.000	ataatTAATgtgtgc
611	V$HOXB6.01	2235	2253	2244	1.000	cgtggtAATTtgttatagc
612	V$HDBP1_2.01	327	345	336	1.000	ccgtgcCCGGccctggtgt
613	V$HEN1.02	3003	3023	3013	1.000	ggagatccaGCTGcgacacaa
614	O$SPT15.01	1120	1136	1128	0.763	atggttaTAAAtatgct
615	V$TEF1.01	2971	2983	2977	1.000	tccCATTccctcg
616	V$HOXB5.01	1895	1913	1904	1.000	gaggaTAATaaactgactt
617	V$CDX2.02	1153	1171	1162	1.000	cctgaaatTTATacctcct
618	O$MTATA.01	2921	2937	2929	1.000	ggctcTAAAtagggagg
619	V$CREB2.01	3719	3739	3729	1.000	agcctaTGACatcagacagga

620	V$OLF1.01	37	59	48	1.000	tggcagTCCCatgagaagctcag
621	V$E2F.03	3473	3489	3481	1.000	gagggGCGCgagctaaa
622	V$COUP.01	2494	2518	2506	0.779	ggggatgggcagAGGCcgggacca
623	V$OC2.01	109	125	117	1.000	ccagatAATCcatgcct
624	V$IRF3.01	242	262	252	0.758	aggggaaaggGACAgaattgc
625	V$NRF1.01	3261	3277	3269	1.000	ggcGCGCacgcccttc
626	V$MEOX1.01	1578	1596	1587	1.000	ctcagatAATTttgcaata
627	V$IPF1.01	2231	2249	2240	1.000	cattcgtggTAATttgtta
628	V$LHX3.02	1450	1472	1461	1.000	tgcactgaaaaaaTAATtctctg

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