The Rho/ROCK GTPase pathway differentially modulates the specification and differentiation of chondrocyte and osteoblast lineages from pluripotent stem cells

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The Rho/ROCK GTPase pathway differentially modulates the specification and differentiation of chondrocyte and osteoblast lineages from pluripotent stem cells

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Dedication

To my parents ….Dr.Mohammed and Thouraya.

Declaration

No part of the work presented in this thesis has been submitted in support of another degree or qualification at this or any other institute of education.

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Abstract

It is well-established that *in vitro* differentiation of Embryonic Stem Cells (ESCs) can recapitulate embryonic development. In this project a novel, step-wise, serum-free differentiation system was developed using specific recombinant growth factors for investigating the differentiation of mESCs, through formation of a primitive streak-like population expressing Brachyury and specification of a subpopulation of mesodermal progenitors expressing both paraxial and lateral plate mesoderm markers. These cells subsequently differentiated efficiently in monolayer cultures to chondrocyte and osteoblast lineages marked by cell morphology, Alcian blue/ALP staining as well as by chondrocyte and osteoblast-specific gene expression.

The role of the Rho kinase (ROCK) pathway in cartilage and bone cell differentiation is controversial. Exposure of ESC cultures to the ROCK inhibitor, Y-27632, at mesoderm enrichment and/or monolayer differentiation phases revealed that continuous exposure to Y27632 modulated differentiation in a developmental phase-dependent manner, with up to a 7-fold and 2-fold increase in chondrogenesis and osteogenesis, respectively. In contrast, temporal exposure of Y27632 favoured chondrogenic over osteogenic differentiation. This was confirmed by qPCR analysis of chondrocyte (Sox9, Sox5, Acan, Coll II, Col X) and osteoblast (Runx2, Osx, ALP, BSP, OC)-specific gene expression. Furthermore, temporal exposure to FGF2 and BMP4 together with phase-specific addition of Y-27632 enhanced differentiation/expansion of hypertrophic chondrocytes and mineralising osteoblasts. Finally, renal capsule grafting studies showed that the mesoderm-derived ESCs mimicked endochondral ossification, which was enhanced by Y27632 treatment.

This study established a novel ESC model system, which generated defined, manipulatable and expandable chondro-osteoprogenitor populations that will provide insights into the molecular basis of bone/cartilage development. Moreover, a phase-dependent inhibition of ROCK signalling modulated early chondro-osteoprogenitor lineage commitment and enhanced cartilage and bone formation. These studies provide a novel targetable pathway for generating specific populations for potential bone and cartilage tissue repair and replacement.

Abbreviations

ATDC5	Chondrogenic cell line
BMP	Bone morphogenic protein
Bry	Brachyury
CCE cell line	XY stem cell line EK. CCE derived from the 129/Sv/Ev strain
cDNA	Complementary deoxyribonucleic acid
d	day
EBs	Embryoid Bodies
ECO	Endochondral ossification
ECM	Extracellular matrix
FACS	Fluorescence activated cell sorting
Flk-1	Fetal liver kinase 1 (VEGFR2/KDR)
FGF	Fibroblast growth factor
hESCs	Human ESCs
h/hs	hour/hours
H&E	Haematoxylin & Eosin
ICM	Inner cell mass
iPS	Induced pluripotent stem cell
MC3T3-E1	Murine osteoblast cell line
mESCs	Murine ESCs
Mesp1	Mesoderm posterior 1
Oct4	octamer-binding transcription factor 4 (Pou5f1, POU domain,class 5, transcription factor 1)
Pdgfrα	Platelet-derived growth factor receptor α
ph	phase
PS	Primitive streak
Pax1	Paired box 1

Abbreviations (cont'd)

Nanog	Nanog homeobox
qPCR	Quantitative reverse transcription Polymerase chain reaction
RT-PCR	Semi-quantitative reverse transcription Polymerase chain reaction
Rho/A	homolog gene family, member A
Runx2	Runt-related transcription factor/ core binding factor-α (CBFα)
RNA	Ribonucleic Acid
SFD	Serum free differentiation media
SFD+S	Serum free differentiation media plus supplements
Sox	SRY (sex determining region Y)-box
SCID	Sever combined Immunodeficiency
TFG-β	Transforming growth factor-beta
VEGF	Vascular endothelial growth factor
Y27632	Rho-associated protein kinas (ROCK inhibitor)
TUNEL	Terminal Deoxynucleotidyl transferase dUTP Nick End labelling
Histo-clear™	National Diagnostic, HS202
Gata-1	GATA-binding factor 1
Nkx2.5	Homeobox protein Nkx-2.5
Tcf15	Transcription factor 15 (basic helix-loop-helix)
Tbx18	T-box18
Nkx3.2	NK3 homeobox 2
Msgn1	Mesogenin 1
Tbx6	T-box 6
Meox1	Mesenchyme homeobox 1
Mesp1	Mesoderm posterior

Abbreviations (cont'd)

Foxf1a	Forkhead box F1
Prrx1	Paired related homeobox 1
Col II	Collagen type II
Prg4	Proteoglycan 4
Osterix (Sp7)	Transcription factor Sp7
Col I	Collagen type I
BSP	Integrin-binding sialoprotein (IBSP)
OC	Osteocalcin (Bone gamma-carboxyglutamic acid-containing protein (BGLAP))
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
-/-	Absence of Y27632 in phase II/ absence of Y27632 in phase III
+/-	Presence of Y27632 in phase II/ absence of Y27632 in phase III
-/+	Absence of Y27632 in phase II/presence of Y27632 in phase III
+/+	Presence of Y27632 in phase II/ presence of Y27632 in phase III

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Chapter 1 Introduction

1.1 Part A
1.2 Embryonic development of cartilage and bone

Skeletogenesis in higher vertebrates is a process which involves the distinctive development of cartilage, bone and joint tissues (Olsen et al. 2000). Both cartilage and bone tissues are characterised by distinct cellular and extracellular matrix components (Yang 2013). However, the process of embryonic development of cartilage and bone has similarities, which involves three phases: first, the migration of osteogenic/chondrogenic precursors to the site of future skeletogenesis; second, the mesenchymal–epithelial interactions that leads to condensation (or aggregation) of the mesenchymal cells and finally the third phase of mesenchymal cells differentiation into either the chondrogenic or osteogenic lineages (Olsen et al. 2000; Lefebvre & Bhattaram 2010;Yang 2013).

The osteogenic/chondrogenic precursor cells (or skeletogenic cells) arise from mesenchymal-type stem cells (MSCs) (Olsen et al. 2000; Lefebvre & Bhattaram 2010) that are formed from embryonic cells known as neural crest or mesoderm cells (Gilbert, 2000; Keller & Nieden, 2011; Olsen et al., 2000). The majority of the skeleton is formed of mesoderm-derived cartilage and bone. Mesodermal cartilage and bone develop from paraxial mesoderm and lateral plate mesoderm. Paraxial mesoderm gives rise to vertebrae, ribs, while lateral plate mesoderm, gives rise to limb joint and growth plate bones (Keller & Nieden 2011) as will be discussed further in the next section 1.3. On the other hand, the craniofacial skeleton largely develops from neural crest cells (Jiang et al. 2002; Santagati & Rijli 2003; Morriss-Kay & Wilkie 2005; Keller & Nieden 2011) (Fig.1.1). The next section will describe the embryonic development of both paraxial mesoderm and lateral plate mesoderm development.

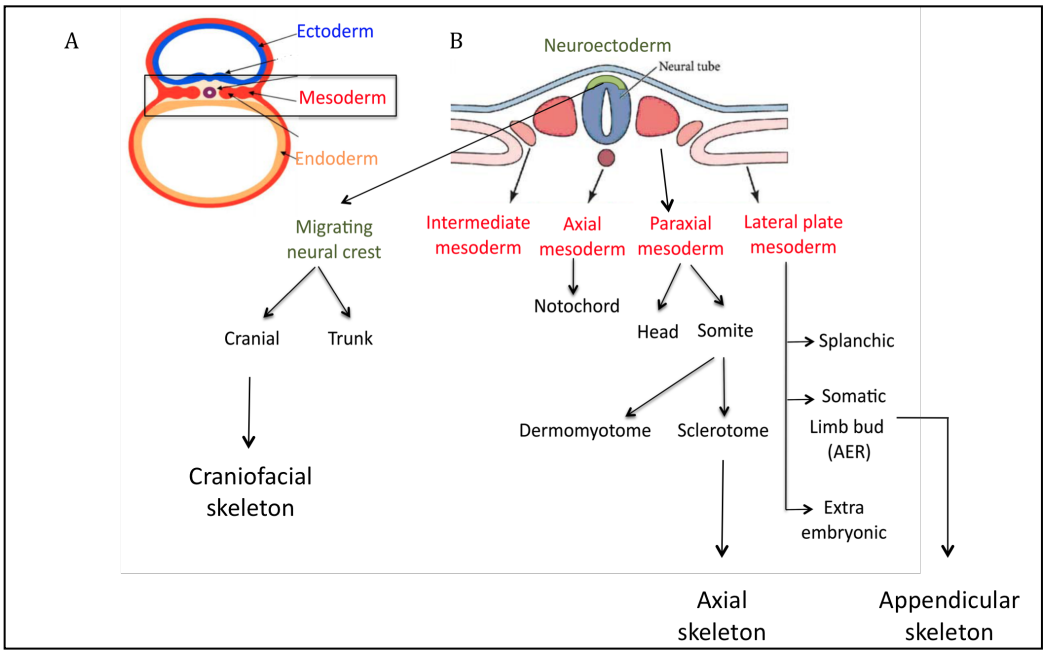


Figure 1.1: A schematic diagram showing the development of sclerotome and limb bud mesenchyme cells, which are the skeletal precursors of cartilage and bone tissues.

A) A cross-section of a gastrulating mouse embryo (after gastrulation at day 8 of development) showing the 3 germ layers; ectoderm (blue), mesoderm (red), and endoderm (orange). B) A cross section of the embryo showing the mesoderm derivatives in a gastrulating embryo. In addition, the migration of neural crest cells, following neuralectoderm formation, the origin of craniofacial bone (modified from Gilbert 2000).

1.3 Embryonic development of mesoderm-derived cartilage and bone

Embryogenesis involves a process called gastrulation during which the formation of three germ layers occurs; ectoderm, mesoderm and endoderm and form the body plane. The beginning of gastrulation is marked by the formation of the primitive streak (PS) at E6 of mouse development (Tam & Behringer 1997; Arnold & Robertson 2009). The PS is a transient structure, located at the posterior part of the embryo, through which epiblast cells ingress and migrate, and differentiate to mesoderm and endoderm (Gilbert 2000). Ectodermal cells are located in the anterior part of the embryo and do not migrate through the PS (Tam & Beddington 1987). The significance of the formation of the PS and the 3 germ layers have been implemented in the *in vitro* differentiation of ESC, and this issue will be further discussed in section 1.10.1.

1.3.1 Mesoderm

Mesoderm is the middle germ layer that occupies an intermediate position between the ectoderm and endoderm germ layers (Gilbert 2000). The mesoderm is divided into 4 regions; axial mesoderm (chordal), paraxial mesoderm, which forms along side the notochord “axial mesoderm”, (somatic dorsal), intermediate mesoderm, and lateral plate mesoderm (away from the notochord) (Gilbert 2000). As mentioned in the previous section, MSCs undergo a stage of condensation then differentiate to either cartilage or bone, which is predetermined by a stage of skeletal patterning and positional identification of the axial and appendicular skeleton (Olsen et al. 2000;Yang 2013) (Fig.1.2).

1.3.2 Paraxial mesoderm

Axial bone and ribs (most of the axial skeleton) develop from paraxial mesoderm, which gives rise to somites (precursors of vertebrae) in a process called somitogenesis (Gilbert 2000). The anterior part of paraxial mesoderm (un-segmented mesoderm is known as ”presomitic mesoderm (PSM)”), which develops into somites (segmented mesoderm) (Kulesa & Fraser 2002a; Tam & Tan 1992). The segmentation of the somites occurs on both sides of the neural tube. Later on, mesenchymal somites differentiate into dermomyotomes and sclerotomes (Aulehla & Pourquié 2010). Each somite is comprised of two parts; rostal (anterior) and caudal (posterior) which are determined prior to somite development (Saga & Takeda 2001). The mechanism of differentiation occurs under the control of various signalling pathways (Aulehla & Pourquié 2010). Somite formation is regulated in part by the transcription factors paraxis (Tcf15) and Tbx18, at which stage their expression coincides with paraxial mesoderm induction (Burgess et al. 1996; Bussen et al. 2004). Tbx6 is required for PSM specification (Chapman & Papaioannou 1998) while Msgn1 is required for pre-somitic mesoderm maturation. Later on, specification of the sclerotome from somites is marked by the expression of Meox1 (Rodrigo 2003; Rodrigo et al. 2004; Mankoo et al. 2003), Pax1 (Dietrich et al. 1993; Rodrigo 2003) and Nkx3.2 (also known as Bapx1) (Tribioli et al. 1997; Tribioli & Lufkin 1999; Hartmann 2009) (Fig.1.2).

1.3.3 Lateral plate mesoderm

The lateral plate mesoderm gives rise to craniofacial skeletal structures, the limb skeletal elements (appendicular skeleton), the sternum (part of the axial skeleton), and non-skeletal structures such as the hematopoietic and cardiovascular tissues (Lawson et al. 1991; Kinder et al. 1999; Olsen et al. 2000). The lateral plate mesoderm consists of dorsal somatic and ventral splanchnic layers. It lines the paraxial mesoderm as two mesenchymal sheets separated by the coelom (Fig. 1.1A). The splanchnic layer surrounds the endodermal gut tube and forms smooth muscle and connective tissue of the digestive organs. Both Mesp1 (Saga et al. 1996; Saga et al. 1999; Takahashi et al. 2005) and Foxf1a (Mahlapuu et al. 2001; Ormestad et al. 2004) are two of the characteristic markers of lateral plate mesoderm (Fig. 1.2). Appendicular bone develops from limb mesenchyme, originating from somatic lateral plate mesoderm (Aulehla & Pourquié 2010; Olsen et al. 2000; Yamaguchi et al. 1993). During limb bud development, mesenchymal cells are controlled by the apical ectodermal ridge (AER)–FGF reciprocal signalling. The mechanism of patterning and differentiation occurs under the control of various signalling pathways: Wnt, BMP and FGF (Aulehla & Pourquié 2010; Yu & Ornitz 2008). Prx1 has been demonstrated, by using a Prx1-cre transgene, to mark limb bud mesenchymal cells, which are undifferentiated and later on give rise to a subpopulation of Sox9-expressing cells. Prx1 is also expressed in the periosteum later in development (Martin & Olson 2000) (Fig.1.2).

In conclusion, mesoderm-derived cartilage and bone tissue development arise from either paraxial or lateral plate mesoderm formation depending on predetermined stage of skeletal patterning and positional identification. Various signalling pathways control the process of mesenchymal progenitor cells of both cartilage and bone lineages. This process is also marked by expression of several gene markers. These signalling pathways and gene markers will be of eminent importance for studying the *in vitro* ESC-derived chondrocyte and osteoblast differentiation, thus recapitulating embryonic development as will be discussed in sections 1.5 and 1.6.

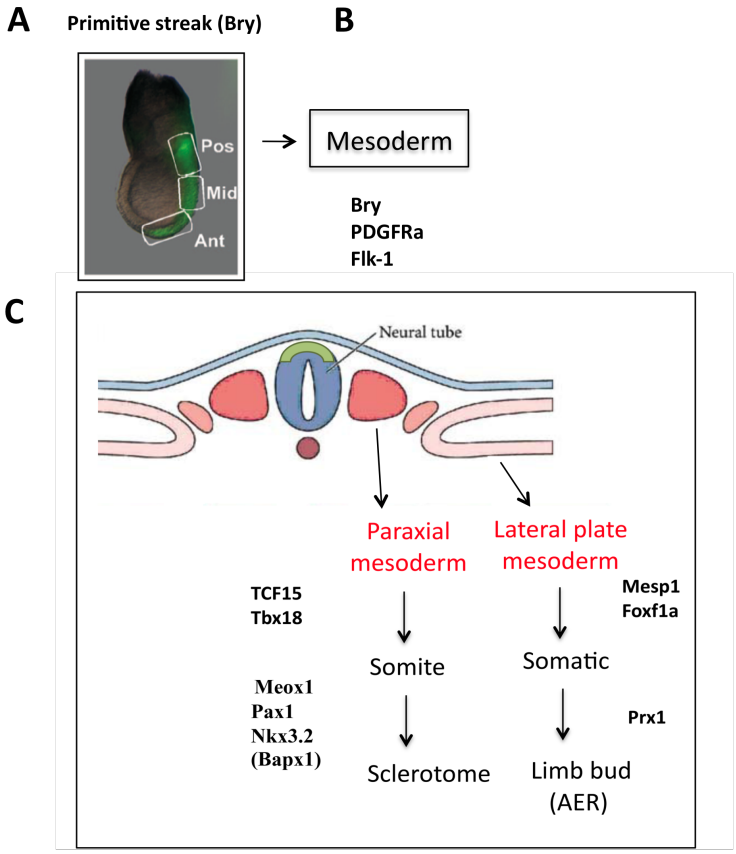


Figure 1.2: A schematic diagram showing selected transcriptional regulation of sclerotome and limb bud mesenchyme cells development, which are the skeletal precursors of cartilage and bone cells.

A) A 7.5 mouse GFP-Bry embryo expressing GPF in the primitive streak (PS), with the anterior (ant), middle (mid), and posterior (post) regions of the PS shown in white boxes (Gadue et al. 2006). B) Bry, Pdgfrα, and Flk-1 are expressed during early/multipotent mesoderm differentiation. C) Tcf15, Tbx18, Meox1, Pax1 and Nkx3.2 are expressed during paraxial mesoderm (PAM) differentiation, while Mesp1, Foxf1a and Prx1 are expressed during lateral plate mesoderm (LPM) formation and differentiation (modified from Gilbert 2000).

1.4 Process of bone formation (osteogenesis)

Once mesenchymal cells reach the required location in the skeleton (i.e. axial or appendicular skeleton tissue), the process of cartilage and bone differentiation occurs, whereby mesenchymal cell condensation occur and common osteo-chondroprogenitor cells differentiate toward either the chondrocyte or osteoblast lineages via one of the two processes of bone ossification; intramembranous or endochondral ossification according to the positional identity of the future bone which occurs along with the specific skeletal patterning process (Hall & Miyake 2000; Yang 2013). In the intramembranous process mesenchymal cells differentiate directly to bone. While in in the endochondral process mesenchymal progenitor cells differentiate into chondrocyte (in a process called chondrogenesis), forming a cartilaginous template. Cells surrounding the template are called the perichondrium, which later changes identity sequentially to be called the periosteum and gives rise to osteoblast precursors. Concurrently, osteogenesis occurs, whereby blood vessel invasion and osteoblast precursors replace the calcified cartilage matrix and lay down bone matrix, which becomes mineralised (Komori 2010; Hartmann 2009; Lefebvre & Bhattaram 2010) (Fig. 1.3).

1.4.1 Chondrogenesis

During chondrogenesis, characteristic changes occur at each stage of cartilage differentiation during the longitudinal growth of bone. Cartilage tissue consists of chondrocytes and characteristic extracellular matrix. Chondrocytes are characterised by a round or polygonal morphology, which express the “Sox trio” (Sox9, Sox5 and Sox6) and secrete extracellular matrix rich in collagen type II (hereafter, as Col II) and aggrecan (hereafter, as Acan), transcriptional regulation will be discussed in section1.5. Then chondrocytes proliferate and expand for elongation and lengthening of bone after which they undergo hypertrophy and mineralisation prior to bone formation to replace the cartilage matrix. Hypertrophic chondrocytes are characterised as enlarged chondrocyte cells and secret collagen type X (hereafter, as Col X) (Wezeman 1998), express matrix metalloproteinase 13 (MMP13), ALP, Runx2 and Ihh (Komori 2010; Pitsillides & Beier 2011; Raggatt & Partridge 2010; Hartmann 2009) (Fig. 1.3).

1.4.2 Osteogenesis

During osteogenesis, characteristic changes occur at each stage of bone differentiation. In general, bone tissue consists of cells, an organic and an inorganic (hydroxylapatite) extracellular matrix. The cellular component is composed of 3 cell types: osteoblasts (bone-forming cells), osteocytes (engulfed osteoblast in mineralised bone matrix) and osteoclasts (bone-resorbing cells). Osteoblasts, in particular, are characterised by a cuboidal morphology, which express Runx2, Osterix (herein Osx) and secrete extracellular matrix proteins rich in collagen type I (herein Col I), ALP, and as osteoblasts mature they upregulate the expression of other proteins such as Bone Sialoprotein (BSP) and Osteocalcin (OC) (Malaval et al. 1999; Aubin and Triffitt 2002; Boskey & Robey 2013; Olsen et al. 2000; Hartmann 2009; Goldring 2012). Osteogenesis is a multistep process that is controlled by a cascade of molecular events, which will be discussed in the next section. Any defects in the process of cartilage or bone formation leads to altered development of cartilage and bone tissues which may result in skeletal deformation and growth retardation (Pitsillides & Beier 2011; Woods et al. 2007) (Fig. 1.3).

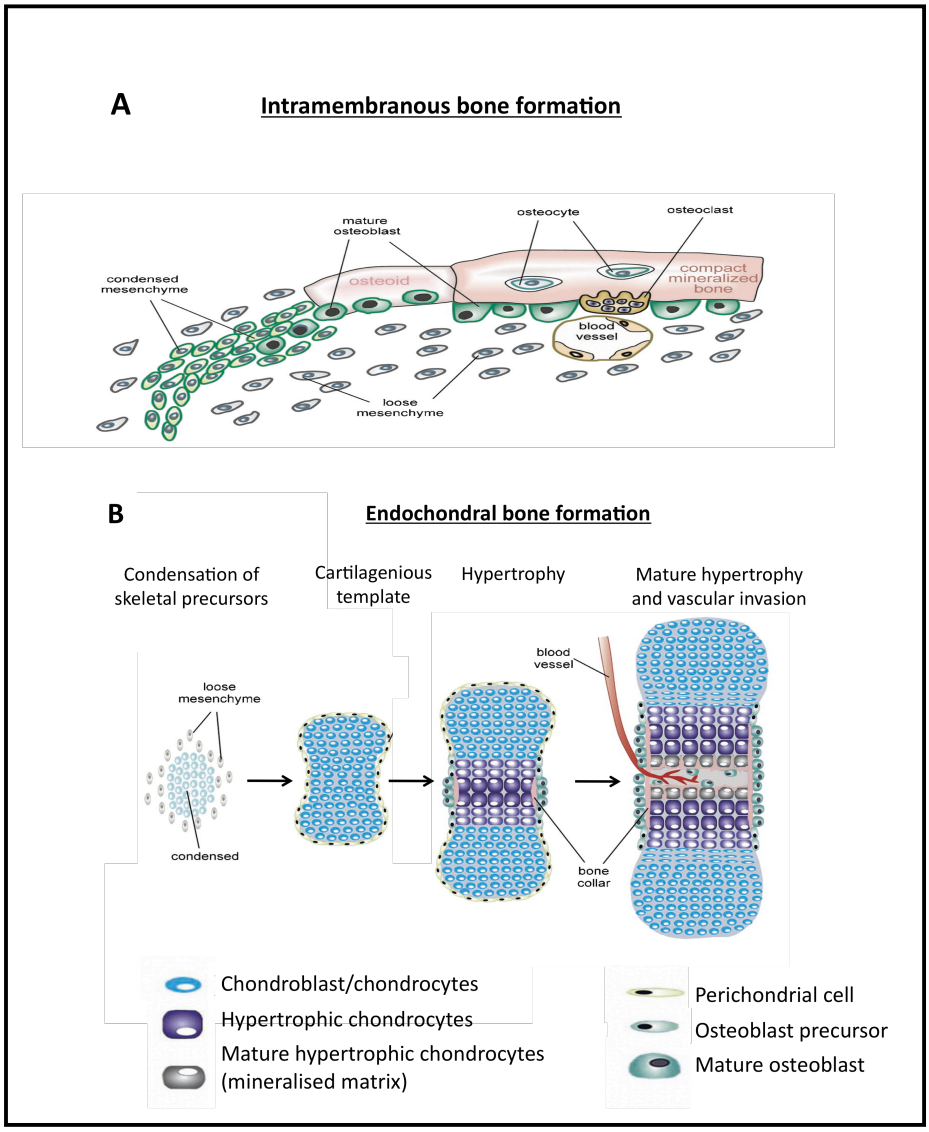


Figure 1.3: A diagram showing intramembranous and endochondral ossification

A) Intramembranous ossification occurs in craniofacial skeleton (flat bones of the skull) and lateral halves of the clavicle bone. Bone formation occurs by mesenchymal stem cell condensation and direct differentiation into osteoblasts (bone forming cells), which lay down bone matrix without a cartilage template stage. B) Endochondral ossification occurs in the axial and appendicular skeleton; long bones and vertebrae. A cartilage template (anlagen) precedes the bone formation stage (mature osteoblast), during which cells mature from chondrocyte to mature hypertrophic cells (modified from Gilbert 2000 and Hartmann 2009).

1.5 Transcriptional control in cartilage and bone; lineage specification and differentiation

The commitment and differentiation to cartilage and bone lineages is regulated by characteristic changes in molecular activity and gene expression as well as in cell shape and ECM synthesis (Hall & Miyake 2000; Hartmann 2009; Raggatt & Partridge 2010; Keller & Nieden 2011). The transcriptional regulation that controls chondrogenesis (cartilage formation) and osteogenesis (bone formation) were identified based on the phenotypic changes seen in human diseases and in knockout or genetically manipulated mice (Akiyama et al. 2002; Lefebvre & Smits 2005; Akiyama 2008; Karsenty et al. 2009) (Fig. 1.4).

1.5.1 Sox9

Sox9 expression is essential for the commitment to the chondrogenic lineage and the formation of mesenchymal condensations (Bi et al. 1999). It is expressed in chondroprogenitors and chondrocytes and not in hypertrophic cartilage (Akiyama et al. 2002) or mature osteoblasts (Akiyama et al. 2005). Furthermore, conditional targeting of Sox9 genes using the Cre recombinase-loxP recombination system (Prx1-Cre transgene) demonstrated that the Sox9-expressing cells originate from an undifferentiated limb bud population during limb bud development in the embryo, suggesting that Sox9 is required for mesenchymal condensation. In addition, using the Col2a1-Cre transgene, inactivation of Sox9 at, and after mesenchymal condensation, stopped the differentiation of condensed mesenchymal cells at the condensation stage and impaired chondrocyte proliferation and maturation (Akiyama et al. 2005).

1.5.2 L-Sox5 and Sox6

In cooperation with Sox9, L-Sox5, a large isoform of Sox5 (hereafter, referred to as Sox5), and Sox6, also regulate chondrogenesis (Lefebvre et al. 1998; Smits et al. 2001). The expression of both Sox5 and 6 is downstream of Sox9 expression. Inactivation of Sox5 and 6 does not prevent chondrogenic differentiation, however, causes a reduction in the expression of Col2a1, Col9a2 and Col11a2 (Smits et al. 2001). Sox5 and Sox6 are expressed along with Sox9 in all pre-cartilaginous condensations and cartilage elements, however, their presence is not observed in mesenchymal condensations (Akiyama et al.

2005; Akiyama 2008). Sox5 and Sox6 cooperate together with Sox9 to activate the Col2a1 enhancer (Lefebvre et al. 1998).

The combined expression of Sox5, Sox6 and Sox9 (the SOX Trio) is the master regulator in the chondrogenic differentiation process. Overexpression of Sox9, Sox5 and Sox6 in cultured cells has been shown to induce chondrogenesis (Ikeda et al. 2004). In addition, transgenic mice in which Sox9 was ectopically expressed in limb bud mesenchyme using the Prx1 promoter exhibited ectopic cartilage formation in association with the induction of ectopic Sox5 and Sox6 expression without any patterning defects in limb bud development (Akiyama et al. 2005).

Sox9 expressing cells are not only progenitors for cartilage but also for bone differentiation during the process of endochondral bone formation (Akiyama et al. 2005). Sox9 expressing cells showed, by using Sox9-Cre;R26R mice, to be common progenitor cells for not only chondrogenic, but also for Runx2 expressing osteogenic progenitors hence indicating a bi-potential progenitor population stage. This observation has also been confirmed in Osx^{flox}/LacZ;Sox9-Cre mice. Interestingly, Sox9 is also expressed in the embryonic development of other lineages than cartilage (Cheung & Briscoe 2003; Blache et al. 2004; Vidal et al. 2005).

1.5.3 Runx2

The Runx2 transcription factor (the runt family) is required for osteogenesis initiation for both endochondral and intramembranous bone formation. Mice lacking Runx2 do not develop osteoblasts and fail to form hypertrophic chondrocytes leading to a cartilaginous skeleton, which does not mineralise (Komori et al. 1997; Otto et al. 1997; Ducy et al. 1999). Ducy et al. (1999) established that overexpression of Runx2 in non-osteoblastic fibroblasts was sufficient enough to induce the expression of osteoblastic markers such as type I collagen, bone sialoprotein (BSP), osteocalcin (OC) and osteopontin (OP) (Ducy et al. 1999). By interacting with many transcriptional activators and repressors and other co-regulatory proteins, Runx2 has been shown to regulate the expression of osteoblast-specific markers (Col1, ALP, OPN, osteonectin ON and OC) in either a positive or a negative manner (Harada et al. 1999).

Runx2 is essential for the proper function of osteoblasts and bone matrix formation (Ducy et al. 1999).

Furthermore, Runx2 is also important in the late stages of chondrocyte differentiation and hypertrophy (Inada et al. 1999; Kim et al. 1999; Takeda et al. 2001). Runx2 overexpression studies showed that under the control of the Col2a1 promoter, Runx2 overexpression accelerates chondrocyte maturation and Col10a1 (hypertrophic cartilage marker) expression in mice, while the dominant-negative Runx2 under the same promoter showed the opposite result (Ueta et al. 2001). Runx2 is also expressed in the osteochodroprogenitor population, but Sox9 expressing cells precede Runx2 expressing cells, during limb bud development in the embryo (Akiyama et al. 2002; Akiyama et al. 2005). Similar to Sox9 expression, Runx2 expression is not required at maturation stage of osteogenesis (Komori 2010).

1.5.4 Osterix

Further on in the osteogenic differentiation process, the expression of osterix (specificity protein-7 (SP7)) (hereafter, referred to as Osx), is required downstream of Runx2 and is in fact a target of Runx2 (Nakashima et al. 2002). Osx is a zinc finger transcription factor which has been shown to control the continuation of osteogenesis towards matrix deposition and mineralisation in both intramembranous and endochondral bones. Osx is responsible for the activation of OC and Col1a1 genes. Runx2-null mice, do not express Osx, but show ectopic cartilage formation and express chondrocyte markers, such as Sox9 and Col2a1 with no bone formation (Nakashima et al. 2002). Furthermore, Osx, is also required in chondrogenenic differentiation whereby chondrocyte-specific Osx conditional knockout using Col2a1-Cre resulted reduction in chondrogenic markers expression and impaired endochondral bone formation (Oh et al. 2012).

1.6 Signalling pathways controlling cartilage and bone lineages differentiation

Beside the transcriptional control, cartilage and bone differentiation are mediated by paracrine signalling pathways such as Bone Morphogenetic Proteins (BMPs) and Fibroblast Growth Factors (FGFs). These signalling pathways are involved in regulating embryonic development and the chondrogenic and osteogenic fate decisions (Hartmann & Tabin 2000; Karsenty & Wagner 2002; Lin & Hankenson 2011) and will be further investigated in this thesis (Fig 1.4).

1.6.1 BMP signalling

BMPs are members of the TGFβ super-family. The connection of BMP with osteogenesis has been first demonstrated when Urist (1962) proved that BMP induced ectopic bone formation in mice. Based on mutation studies, BMPs regulate chondrogenesis and osteogenesis (Bandyopadhyay et al. 2006; Lin & Hankenson 2011). For example, knockout experiments of BMP2 and BMP4 showed a requirement of BMP2 and BMP4 signalling for maturation and completion of osteoblast differentiation whereby osteogenic differentiation is terminated by the loss of BMP2 and BMP4 ligands (Bandyopadhyay et al. 2006). Furthermore, BMP signalling has been also demonstrated to direct MSC commitment towards the osteogenic lineage over the myogenic and adipogenic lineages (Gimble et al. 1995; Hogan 1996; Lin & Hankenson 2011). BMP signalling showed *in vitro* to target Runx2 whereby BMP2 upregulated Runx2 and ALP expression in human bone marrow cells (Gori et al. 1999).

1.6.2 FGF signalling

The FGF gene family is composed of 23 members that bind to FGF tyrosine kinase receptor isoforms (Fgfr). The FGF-family polypeptides play a critical role in regulating bone formation (Marie et al. 2012; Long & Ornitz 2013a). FGF1 and FGF2 were the first FGF ligands to be purified and defined as mitogenic factors of fibroblasts grown in culture (Gospodarowicz & Moran 1975). Mice lacking FGF (2,18, and 23) have demonstrated skeletal phenotypes (Degnin et

al. 2010). FGF signalling is required in limb bud development whereby FGF2 beads showed to replace function of AER (Fallon et al. 1994; Taylor et al. 1994).

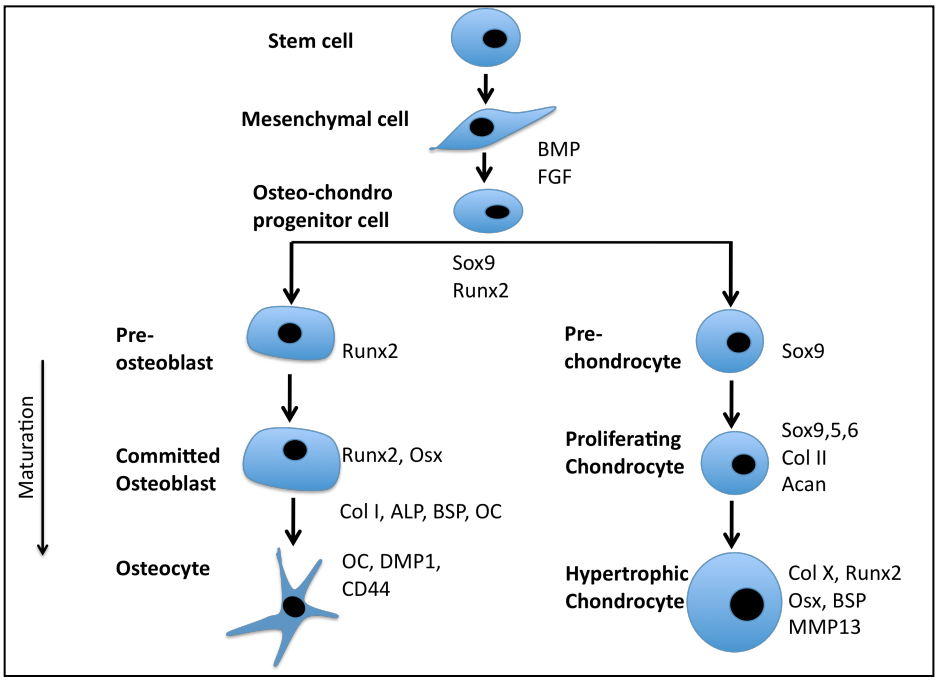


Figure 1.4: A differentiation scheme showing key transcriptional factors and signalling pathways controlling the different stages of bone and cartilage specification and differentiation/maturation.

Differentiation of Mesenchymal cells (MSCs) to chondro-osteoprogenitor cells which commit to either osteoblast or chondrocyte lineages under the control of cartilage and bone specific transcriptional factors (Sox9, 5, and 6, and Runx2 and Osx. respectively. In addition, paracrine signaling pathways (BMP, FGF) control MSC differentiation. Each stage of differentiation shows the expression of cartilage and bone specific phenotypic genes. Collagen type I (Col I), Alkaline phosphatase (ALP), Bone Sialoprotein (BSP) and Osteocalcin (OC), Dentin matrix acidic phosphoprotien 1 (DMP1)) for osteogenic lineage, and Collagen type II (Col II), aggrecan (Acan) and Collagen type X (Col X), Runx2, osterix (Osx), BSP, metalloproteinase (MMP13)) for chondrogenic lineage. (Modified from Long 2012 and Liu et al. 2013).

1.7 ESC differentiation to cartilage and bone

Stem cells are responsible for the development of an organism and the maintenance of organs and tissues throughout life. They have the ability to renew themselves (self-renewal) and to produce daughter cells with the potential to differentiate into different cell types with restricted properties (Blau et al. 2001; Smith 2006; Murry & Keller 2008). In this thesis, I will describe two general types of stem cells; adult and embryonic stem cells. While the latter will be the basis of this project as will be discussed in the later sections.

1.7.1 Adult (Somatic) stem cells

Adult stem cells are committed stem cells found in many organs and differentiated tissues. They have a limited capacity for self-renewal and proliferative potential. They can be multi-potent, but their capacity is normally limited to cell types in the organ of origin (Smith 2006). The most studied Adult Stem Cells that is relevant to this thesis are Mesenchymal Stem Cells (MSCs), described below.

1.7.2 Mesenchymal stem cell (MSCs)

The work of Friedenstein and Owen in the 1960's led to the discovery of non-hematopoietic stem cells in the bone marrow, and these were designated bone marrow stromal stem cells (BMSC) (Friedenstein et al. 1978; Owen & Friedenstein 1988). These cells are clonogenic, multipotent and have the capacity to differentiate *in vitro* and *in vivo* into mesenchymal tissues, such as osteoblasts, chondrocytes and adipocytes. These were later termed as MSCs or skeletal stem cells and were suggested to differentiate to a wide range of cell types cardiomyocytes, neurons, and astrocytes *in vitro* and *in vivo* (Friedenstein et al. 1978; Bianco et al. 1998; Pittenger 1999; Y. Jiang et al. 2002; Bianco et al. 2008; Caplan 2010; Salem & Thiemermann 2010). Although MSCs are found in tissues other than bone marrow, such as adipose tissue, muscle, periosteum, dental tissues and perivascular mesenchymal cells (pericytes) (Sacchetti et al. 2007; Hipp & Atala 2008; RodriguezLozano et al. 2011).

They are all generally considered to be a rare, heterogeneous stem cell population, with a limited capacity for self-renewal and demonstrated to lose clonal mulitpotency (Bianco et al. 1998; Jiang et al. 2002). In addition, MSCs lack unique surface markers, making it difficult to distinguish progenitor cells from specific cell types and thus track their differentiation.

However, some specific cell surface antigens expression have been used as minimum criteria to isolate MSCs, as defined by the International Society for Cellular Therapy (Dominici et al. 2006): CD29, CD44, CD56, CD73, CD90, CD105, CD271 and MSCA-1, as well as In addition, Stro-1 to characterise osteoprogenitor cells (Gronthos et al. 2003; Dominici et al. 2006; Battula et al. 2009; Abdallah & Kassem 2007). These markers are characterised based on plastic “adherence selection“ (Dominici et al. 2006; Salem and Thiemermann 2013). However, there are obstacles regarding methods of culturing and heterogeneity of populations (Salem & Thiemermann 2010).

1.7.3 Embryonic Stem Cells (ESCs)

ESCs have an unlimited capacity for self-renewal and are pluripotent (Evans & Kaufman 1981). The term pluripotency means that these particular cells have the capacity to differentiate to form all germ layers and differentiate to all tissue cell types, which has been demonstrated both in vitro and in vivo using chimaeric mice by Bradley et al. 1984 (Smith 1992; Gadue et al. 2005; Murry & Keller 2008). ESCs originate from the inner cell mass (ICM), which is a transient structure in the pre-implantation embryo stage called the blastocyst stage (spherical embryo). The ICM is defined as “Pluripotent tissue inside the blastocyst that gives rise to the embryo proper and yolk sac tissue.” It was first isolated from mice (Evans & Kaufman 1981; Evans 2011). Following their isolation in mice, ESCs were then isolated from human embryos (Thomson 1998) and other species (Wobus & Boheler 2005; Wobus & Boheler 2009). Murine ESCs were shown to retain normal chromosomal karyotypes even with multiple passaging, and do not undergo senescence compared to somatic stem cells whereby studies suggest the involvement of maintained telomerase, which is a reverse transcriptase that elongates the ends of the DNA (Günes & Rudolph 2013).

Pluripotent stem cells can be used for gene targeting (Smith 1992) through genetically manipulating cells *in vitro*, which has been proven by over-expression and knockout mice, and producing chimaeric mice to trace a specific gene *in vivo* (Irion et al. 2008). In addition, stem cell studies are used to aid in the screening and testing of chemical products before they are tested on humans (Keller 2005; Irion et al. 2009). In terms of tissue regeneration, ESCs provide unlimited, principally a bulk production of a homogeneous cell source hence overcoming the limitations of MSCs. On the other hand, the disadvantage of ESCs as a model system relies on their ability to form teratoma *in vivo* and the challenge is the ability to control and direct the differentiation of ESCs towards a specific cell lineage. The latter will be discussed further in sections 1.10.

1.7.4 Induced Pluripotent Stem cells (iPSCs)

iPSC reprogramming is a method of engineering or reversing the state of a differentiated somatic cells to an undifferentiated ESC-like state without the use of an embryo. The first iPSCs were generated in 2006 when Takahashi and Yamanaka reported reprogramming of a mouse fibroblast by transient retroviral transduction of cells with four embryonic transcription factors (Yamanaka et al. 2008). iPSC lines have been established from diseased patient cells, such as Duchenne Muscular Dystrophy or Amyotrophic lateral sclerosis (ALS), therefore allowing for studying disease *in vitro* and recapitulating development of a specific cell type/tissue in a dish (Dimos et al. 2008; Park et al. 2008). The behaviour of iPSCs resembles ESCs, in that they are pluripotent, they express pluripotency markers and cause teratoma formation. The unique character of iPSCs is that they convey the potential of providing patient - specific cell-based therapy, as well as a model to study patient - specific diseases (Wu & Hochedlinger 2011).

In summary, ESCs are attractive cell source for advancing in *vitro* differentiation techniques for directed and controlled differentiation under appropriate conditions. This will permit the study of early cell precursors in order to understand specific cell-type lineage commitment (Gadue et al. 2005; Murry & Keller 2008). For this project, ESCs will be used specifically to look into chondro-osteoprogenitor progenitor differentiation, as will be discussed further in sections 1.10.2.

1.8 Mouse ESCs

In this project the focus will be on mouse ESCs. Morphologically, mouse ESCs grow in colonies of small cells with a high nucleus to cytoplasmic ratio (Smith 1992). SSEA-1 (Solter & Knowles 1978) and alkaline phosphatase are characteristic mESC surface markers. Mouse ESCs without feeder cells are maintained in an undifferentiated state (as found in the blastocyst) using leukemia inhibitory factor (LIF) in a serum-free cultures (Williams et al. 1988; Ying et al. 2008).

1.8.1 *In vitro* ESC differentiation methods

There are three methods which have been established to initiate ESCs differentiation (Keller 1995; Murry & Keller 2008) (i) Embryoid Body formation, (ii) co-culture with stromal cells rich with suitable factors, and finally (iii) direct culturing of cells on defined matrices such as collagen type IV (Nishikawa et al. 2007).

The most common method used is the Embryoid Body formation. Embryoid Bodies (EBs) are 3D structures of ESC aggregates in culture. This method allows for the creation of a gastrulation phase *in vitro* (formation of the three germ layers), mimicking the peri-implantation embryo environment, where interactions between various cell types facilitate inductive events and accessing progenitor cells that are not yet committed to any specific cell lineage (Murry & Keller 2008). There are two techniques for EB formation in non-adherent cultures: First, ESCs are plated in a static suspension method using a liquid medium or methylcellulose in a Petri dish. This method prevents the adhesion of ESCs to the surface and allows them to aggregate and form EB structures. The second method, is using a three-step method, where cells are seeded on a

dish lid as “hanging drops” and then condensed by gravitational force so forming uniform EBs which are then transferred to a liquid culture (Keller 1995; Murry & Keller 2008). In this project, EB formation using the static suspension technique was selected in order to expose ESCs to mesoderm induction/and enrichment growth factors during differentiation. This point will be further explained in details in chapter 2 and 3 details.

1.9 ESC differentiation to cartilage and bone via EB culture method

Early studies to understand cartilage and bone differentiation processes have been carried out on primary cells, tumour cells and mesenchymal cell lines (Bellows et al. 1990; Erlebacher et al. 1995; Bellows et al. 2003). These culture models were composed of heterogeneous populations of differentiated and undifferentiated cells and represented relatively late stages of development (Bellows et al. 1990; Duplomb et al. 2007). Later on, researchers have used ESC *in vitro* culture models in order to understand bone and cartilage differentiation from the earliest stages of embryonic development to terminal differentiation stages by differentiating ESCs to EBs first then culturing the created cells in chondrogenic or osteogenic (Buttery et al. 2001; Hegert 2002; Kramer et al. 2000; Kramer et al. 2003; zur Nieden et al. 2003; Kawaguchi et al. 2005b; zur Nieden et al. 2005).

For osteogenic differentiation, the first cocktail used by Buttery et al (2001) consisted of ascorbic acid, β-glycerophosphate and dexamethasone and proved to mineralise osteoblast nodules. The osteogenic media was later on modified by many laboratories by the addition of inducing factors to enhance the differentiation such as compactin (Phillips et al. 2001), retinoic acid, (Phillips et al. 2001; Kawaguchi et al. 2005), 1,25alpha (OH)2 vitamin D_3 ($VitD_3$) (zur Nieden et al. 2003; zur Nieden et al. 2007), BMP2/4 (Kawaguchi et al. 2005) and conditioned medium from the human hepatocarcinoma cell line HepG2 (Hwang et al. 2006). Theses studies examined osteoblast differentiation by mineralisation of bone nodules and bone-specific markers expression. For chondrogenic differentiation, the inducing factors investigated were, for example; TGFβ1/3 and BMP2/4/7, IGF-1 and ascorbic acid (Kramer et al. 2000; C. Hegert 2002; zur Nieden et al. 2005; Y. Hwang et al. 2007). The examination

of chondrogenic media was evaluated by gene expression of chondrogenic genes and Alcian blue staining.

The majority of the studies demonstrated the in vitro potential of ESC to differentiate towards the chondrogenic and osteogenic lineages. However, the reports relied on the presence of serum in the culture conditions, which may lead to an uncontrolled differentiation of ESC and the generation of heterogeneous cell populations. The presence of fetal calf serum (FCS) in the culture medium offered a source of unknown essential cell nutrient (agonist) growth factors as well as inhibitor factors (antagonist). Moreover, a batch-to-batch variability led to variable cell differentiation results. Furthermore, the uncontrolled EB-based differentiation of ESCs induces spontaneous mesoderm along the side with cell types from the other germ layers, which could result in an inefficient differentiation of ESCs to chondro-osteoprogenitor population and incomplete differentiation and commitment of cells to the required cell type lineage (Keller 2005; Nostro et al. 2011; Nakayama & Umeda 2011). These factors therefore make it difficult to produce a reproducible and controllable differentiation system model for stem cell differentiation to bone and cartilage.

In conclusion, these studies indicate the importance and requirement for controlled, directed stem cell differentiation model systems, using a serum free and a chemically defined (signalling molecules) differentiation environment to enrich for novel chondro-osteoprogenitor population, as will be discussed in the next sections.

1.10 ESC differentiation to cartilage and bone differentiation via recapitulation of primitive streak/mesoderm formation

In the last few years, researchers have been focusing on approaches to direct the differentiation of ESCs to a more homogenous population of chondro-osteoprogenitor cells by the manipulation of signalling pathways and purification methods to enrich for mesoderm progenitor populations, thus following a developmental approach, whereby recapitulating mesoderm development and differentiation to mesoderm progenitors allow the access to precursors of both cartilage and bone lineages as mentioned in section 1.3 (Murry & Keller 2008; Kitagawa & Era 2010; Keller & Nieden 2011; Nakayama & Umeda 2011) (Fig. 1.5).

During embryonic development, the formation of mesoderm is controlled spatially and temporally via specific signalling mechanisms including the TGF-β superfamily (TGF-β, BMP, Nodal), FGF, and Wnt signalling pathways (Nishikawa et al. 2007; Irion et al. 2009). Initial studies on mesoderm induction/differentiation have been done using Xenopus and Zebrafish embryos. This basic process is conserved in all vertebrates (Kimelman 2006). Studies of both loss- and gain-of-function in mice reported that Nodal (Conlon et al. 1994; Schier & Shen 2000), BMP4 (Hogan 1996) and Wnt (Yamaguchi 2001) are key pathways in directing this process.

Mesoderm differentiation follows the stage of primitive streak differentiation - as mentioned in section 1.3 - Gadue et al. (2006) established using a mESC culture model that the activation of activin/Nodal, BMP and Wnt signalling pathways are required to recapitulate a primitive-like (PS) cell population *in vitro.* The differentiation of the primitive streak-like cell population could later on be directed towards sub-mesodermal (Lindsley et al. 2006; Gadue et al. 2006; Gouon-Evans et al. 2006; Nostro et al. 2011) as well as endodermal (Gouon-Evans et al. 2006) populations under the control of a specific combination of these signalling factors, i.e. concentration and time. Therefore, through manipulation of the above signalling pathways, it is possible to differentiate ESCs to MSC-like, specifically chondro-osteoprogenitor populations *in vitro* (Keller 2005; Murry & Keller 2008).

1.10.1 Directed ESC differentiation to primitive streak/mesoderm lineage

It has been proven in Xenopus that the concentration gradient of the signalling molecules influences the subpopulation of the germ layer generated (Green et al. 1992; Fukui & Asashima 1994; Kubo et al. 2004). Higher concentrations of Activin A direct cells towards endodermal lineages while low concentrations direct cells towards mesodermal lineages (Okabayashi & Asashima 2003). Activin A acts as a substitute for Nodal, it acts through the same receptor as Nodal (Gadue et al. 2006). In contrast, a BMP concentration gradient demonstrated an opposite effect (Sumi et al. 2008; Nostro et al. 2011) as been demonstrated in mice (McMahon et al. 1998) (Fig. 1.5).

To trace early mesoderm developmental stages in the ESC culture model, a reporter ESC line such as GFP-Bry and subsequent gene expression have been used to trace PS/mesoderm from EB formation *in vitro* (Fehling 2003; Gadue et al. 2006). Craft et al (2013) utilised the GFP-Bry cell line to monitor mesoderm differentiation in mESC cultures. Brachyury (a conserved T box transcription factor) is expressed throughout the primitive streak (Smith et al. 1991) and early mesoderm (Wilkinson 1990; Herrmann & Kispert 1994; Yamaguchi et al. 1999; Kubo et al. 2004). Umeda et al (2012) utilised Mixl-1-GFP hESCs to differentiate chondrogenic progenitors from a mesoderm progenitors. Mixl-1 is also expressed in the PS and early mesoderm. It is a common marker for mesoderm and endoderm (Pearce & Evans 1999; Hart et al. 2002).

Furthermore, several studies revealed that the manipulation of different signalling pathways at specific developmental stages may direct the early stages of mesoderm induction and distinguish it from PS formation (Murry & Keller 2008; Nostro et al. 2008). Surface markers such as the receptor kinase Flk-1 and Pdgfrα (Kataoka et al. 1997; Ema et al. 2006; Murry & Keller 2008; Nostro et al. 2011) have been used to further purify intermediate mesoderm progenitor populations (Kitagawa & Era 2010).

In vitro ESC fate analysis showed that Flk1-positive cells differentiate into lateral plate mesoderm derivatives (Nishikawa et al. 1998; Yamashita et al. 2000; Wang 2006; Kattman et al. 2006) whereas Pdgfrα differentiates towards paraxial mesoderm derivatives (Nakayama et al. 2003; Sakurai et al. 2006), which recapitulates normal embryonic development. Pdgfrα and Flk-1 have been used for purification of chondro-osteoprogenitor populations as will be discussed in the next section. However, more specific markers are required besides Pdgfrα and Flk-1 for further characterisation and purification of chondro-osteoprogenitor populations, such as a Pax-1 sclerotome marker for mature paraxial mesoderm or a Prx-1 positive limb mesenchyme for mature later plate mesoderm (Nakayama & Umeda 2011).

In conclusion, using ESCs *in vitro* as a model for recapitulating PS and lineage commitment provides an opportunity to generate enriched, functional cell types under defined culture conditions via recapitulating the gastrulation phase and demonstrate mesoderm gene expression *in vitro.*

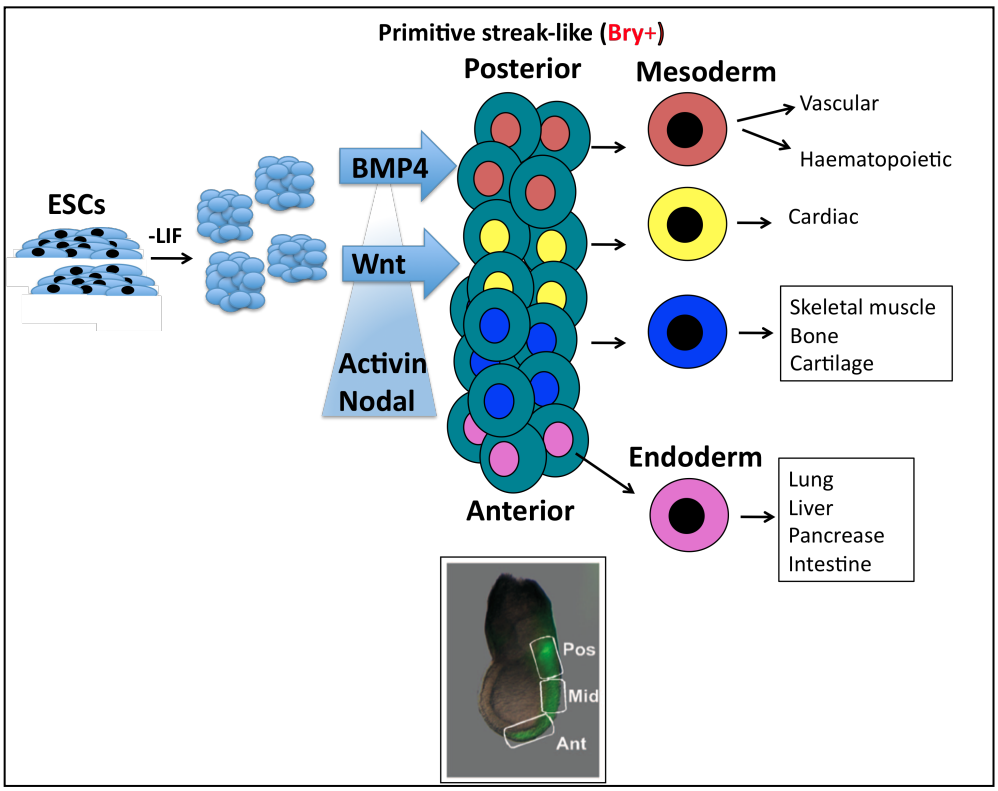


Figure 1.5: A scheme showing differentiation of ESCs using the Embryoid Body model system to recapitulate embryonic development of mesoderm and endoderm *in vitro*.

A scheme showing the differentiation of ESC to EBs after the removal of LIF. A primitive streak (PS)-like population expressing Brachyury (Bry-positive) is generated recapitulating the embryonic development of the PS *in vivo* (inset showing a mouse embryo (E7.25) with GFP-Bry positive PS regions, ant, mid, post. BMP, Activin A, and Wnt signalling pathways induce and enrich for mesoderm and endoderm differentiation. The manipulation of these signalling pathways temporally as well as by concentration directs the differentiation of PS-like cells to either anterior or posterior regions of the PS, providing efficient and reproducible control of the differentiation of specific cell types required (modified from Keller 2005 and Gadue et al 2006).

1.10.2 Directed ESC-derived mesoderm differentiation towards cartilage and bone lineages

As opposed to spontaneous ESC-derived mesoderm differentiation to chondrogenic and osteogenic pathways, recently ESC differentiation has demonstrated mesoderm differentiation in a defined medium to generate controlled and purified populations of a specific cell type. This is by recapitulating primitive streak formation then mesoderm formation (Nishikawa et al. 2007; Murry & Keller 2008; Kitagawa & Era 2010). The following studies were discussed in here mainly in the context of mESCs studies with only some related hESC studies.

1.10.3 Paraxial and lateral plate recapitulation and differentiation

Several studies defined culture conditions for directing the differentiation of mESCs towards paraxial/lateral plate mesoderm and chondrocyte development (Nakayama et al. 2003; Sakurai et al. 2006; Tanaka et al. 2009; Craft et al. 2013). The following studies highlighted the importance of signalling factors; timing and interaction between growth factors as well as the purification methods for enriching specific mesoderm progenitor populations. Nakayama et al. (2003) were the first to use Pdgfrα and Flk-1 as markers for purifying mesoderm intermediate populations, which later showed to acquire chondroprogenitor potential.

Tanaka et al. (2009) proved the generation of (Pdgfrα+) population with chondrogenic potential in a serum free culture condition. The mesoderm induction was through the activation of Activin/Nodal and Wnt signalling with the inhibition of BMP. Interestingly, Umeda et al (2012) demonstrated same results in hESC culture, suggesting that the mesoderm induction protocol used was efficient for paraxial mesoderm-derived chondrogenic differentiation. Furthermore, several studies proposed the differentiation of both cartilage and bone cells using the same mesoderm progenitor population. Sakurai et al (2006) established that a Pdgfrα+ population has both an osteogenic and chondrogenic potential.

However, these Pdgfrα+ populations could originate from either: (1) (Pdgfrα+/ Flk-1+) (bi-potential potential paraxial and lateral plate mesoderm population) (2) (Pdgfrα+/ Flk-1-) which consists mainly of paraxial mesoderm population), but not from (Flk-1+/Pdgfrα-) which consists mainly of paraxial mesoderm population evident by gene expression of osteogenic and vascular gene markers. Furthermore, Sakurai et al (2009) proved in a serum free culture conditions that the induction by continuous BMP4 signalling formed cartilage and bone both *in vitro* and *in vivo*, in quadriceps femoris muscles of immunodeficient mice.

1.10.4 Three phase differentiation

Very recently, using mESCs, Craft et al., (2013) demonstrated paraxial mesoderm derived chondrocyte potential using a stage-specific manipulation of the same signalling cocktail utilised in the Tanaka et al. (2009) study, in a serum-free culture (i.e. inhibition of BMP with the activation of Activin/Nodal and Wnt signalling). The mesoderm induction generated a Pdgfrα+ population more efficiently than cultures containing BMP. This 3-stage signalling manipulation that Craft et al. (2013) utilised has been applied in hESCs in a similar approach and demonstrated to generate ~74-97% of Sox9-expressing cells, thus increasing the efficiency of the chondrogenic differentiation (Oldershaw et al. 2010).

In conclusion, several studies proved the generation of chondrogenic and osteogenic progenitor populations using a developmental approach whereby ESCs recapitulate embryonic development of mesoderm followed by cartilage and bone differentiation. However, there is still controversy for which cocktail of signalling pathways is best for the generation of mesodermal progenitor subpopulation, that might provide improved chondrogenic/osteogenic progenitors for differentiation. Nevertheless, the development of a stage-specific, manipulative system for mesoderm differentiation in a serum-free condition demonstrates a more controlled method for mesoderm differentiation to cartilage and bone lineages.

The later approach will facilitate in addressing developmental questions regarding cartilage and bone specification and differentiation. Therefore, this will provide the base for this thesis for further studying of the molecular events controlling lineage specification and differentiation towards both cartilage and bone lineages, specifically related to ROCK signalling pathway as will be discussed in the second part of this chapter.

1.11 Part B

1.12 Rho/ROCK signalling pathway

The Rho (Ras homologous) family of signalling molecules is part of the Ras superfamily of small guanosine triphosphatases “GTPases” (Wennerberg & Der 2004; Wennerberg et al. 2005). The best characterised Rho GTPase signalling subfamilies include: Rho “including 3 isoforms: A, B, and C” (RAS homologue gene family member), Rac1, and Cdc42 (cell division cycle 42) (Matsui et al. 1996; Amin et al. 2013).

Rho GTPase signalling is involved in several cellular signal transductions pathways to the cell through many cell surface receptors, including tyrosine/serine/threonine kinase receptors, G-proteins, and integrins (Assoian & Schwartz 2001; Boudreau & Jones 1999; Whitehead et al. 2001; Riento & Ridley 2003; Mueller et al. 2005; Heasman & Ridley 2008; Ridley 2013)(Fig.1.6). For controlling cellular functions, they behave as a molecular switch by cycling between an inactive, GDP-bound state, and an active, GTP-bound state. This is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Riento & Ridley 2003; Mueller et al. 2005; Bustelo et al. 2007; Heasman & Ridley 2008; Ridley 2013) (Fig. 1.6).

Rho GTPases are involved in cell motility, proliferation and apoptosis by acting on the cytoskeleton and microtubules. Moreover, they are also involved in cell cycle progression and differentiation processes through their effects on gene expression (Wennerberg & Der 2004; Heasman & Ridley 2008). The activation of Rho (GTP-bounded) subsequently translocates to specific subcellular locations to activate many downstream targets, one of which is Rho-associated kinase (ROCK) (Riento & Ridley 2003; Jaffe & Hall 2005; Heasman & Ridley 2008; Amano et al. 2010) (Fig.1.6). ROCK signalling specifically will be the focus of the following sections in particular in the context of cartilage and bone differentiation.

1.12.1 Rho- associated coiled-coil-forming kinase (ROCK)

ROCK (also known as Rho-kinase/ROK) is an immediate downstream effector molecule of the Rho GTPses subfamily of small GTPases and also one of the best characterised effectors (Leung et al. 1996; Matsui et al. 1996) (Fig. 1.6). It is a serine/threonine protein kinase, which phosphorylates proteins involved in many cellular functions (Leung et al. 1996; Nakagawa et al. 1996). ROCK includes two isoforms, ROCK1 (ROKβ/p160ROCK) and ROCK2 (ROKα), each encoded by different genes (Nakagawa et al. 1996; Riento & Ridley 2003; Mueller et al. 2005; Amano et al. 2010). Mouse knockouts for ROCK1 and ROCK2 were shown to be embryonic lethal due to placental dysfunction and growth retardation (Thumkeo et al. 2003; Mueller et al. 2005). Both ROCK isoforms share an overall identity; 65% in the amino acid sequences and 92% in the kinase domains, having similar substrate specificity (Riento & Ridley 2003; Amano et al. 2010). Although both ROCK isoforms show similarities and are ubiquitously expressed in mouse tissue; however, their mRNA is present at higher levels in specific tissues and may show specific functions, thus suggesting that they might demonstrate different functions. ROCK2 is expressed in higher levels in brain and muscles whereas ROCK1 is present in higher levels in non-neuronal tissues, including liver, lung and testis (Leung et al. 1996; Nakagawa et al. 1996; Hashimoto et al. 1999; Riento & Ridley 2003; Amano et al. 2010).

At present, an understanding of ROCK function is derived mostly from *in vitro* studies. *In vivo* studies have reported the involvement of both isoforms in actin bundling by the analysis of knockout mice: homozygous ROCK1 (Shimizu et al. 2005a) and ROCK2 (Thumkeo et al. 2003), which showed that both knockouts affect epithelial cell motility as evidenced by eyelid closure and fusion of the ventral body wall, due to the formation of omphalocele and open eyes (Thumkeo et al. 2003; Shimizu et al. 2005). ROCK1 or ROCK2 heterozygous mice also had omphalocele and open eyes (Thumkeo et al. 2005). Until now, there are no published reports on germline or somatic inducible and tissue-specific ROCK knockout mouse models (Bustelo et al. 2007; Amin et al. 2013).

ROCK phospholylates targets several proteins and the major targets include: myosin light chain phosphatase “MLCP”, myosin light chain “MLC”, LIM kinases

”LIMKs”, and collapsing response mediator protein2 “CRMP2”. ROCK was first characterised for its role in RhoA-induced stress fibres and focal adhesion by controlling the reorganization of the actin cytoskeleton and regulation of actin myosin contractility by the phosphorylation of myosin light chain (MLC) (Amano et al. 1996; Leung et al. 1996; Somlyo & Somlyo 2000; Riento & Ridley 2003). It promotes the assembly of actomyosin filaments through the phosphorylation and deactivation of the myosin binding subunit of myosin phosphatase (MYPT1) (Kawano et al. 1999), and direct phosphorylation of myosin light chain (MLC) (Amano et al. 1996; Kimura et al. 1996).

ROCK is involved in several cellular functions, such as cell motility, adhesion, invasion, cell size, differentiation, apoptosis and regulating MSC lineage commitment (Riento & Ridley 2003; Sordella et al. 2003; McBeath et al. 2004; Amano et al. 2010) (Fig. 1.6). The inhibition of ROCK has been investigated in part by the use of pharmacological inhibitors, one of which is Y27632 (Amin et al. 2013). This project will be focusing on the effect of Y27632 on cartilage and bone development as will be discussed in the following section.

1.12.2 ROCK inhibitor (Y27632)

The effect of inhibition of RhoA/ROCK signalling has been studied through the use of pharmacological inhibitors such as Y27632 (Fig. 1.6). The pharmacological inhibitor of ROCK, Y27632, (+)-(R)-trans-4-(1-ami-noethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate was discovered by Yoshitomi Pharmaceutical Industries (Ishizaki et al. 2000). It is one of the first identified ROCK inhibitors through its Ca^{2+} antagonistic effect and potential use for hypertension therapy in rats (Uehata et al. 1997) and is an extensively tested ROCK inhibitor *in vitro* (Mueller et al. 2005; Amin et al. 2013). The mechanism of Y27632 relies on targeting the ATP-dependent kinase domains of both ROCK isoforms and competitively inhibits the phosphorylation of various substrates (Ishizaki et al. 2000; Amin et al. 2013). Both ROCK1 and ROCK2 are inhibited by Y27632, with no selectivity, at equimolar concentration. However, Y27632 can also non-specifically inhibit other protein kinases, such as protein kinase-related protein (PRK)-2, at equal potency and inhibits other protein kinases: protein kinase N, and citron kinase, at higher concentrations (Davis 2011; Zhou et al. 2011; Amin et al. 2013).

Several reports have described potential therapeutic effects of ROCK inhibitors, including Y27632 in cases involving the central nervous (Mueller et al. 2005) and cardiovascular systems (including spinal cord injury, vasospasm, hypertension, atherosclerosis, and myocardial hypertrophy) (Zhou et al. 2011), diabetic nephropathy (Komers 2011), and cancer invasion and metastasis (Itoh et al. 1999; Li et al. 2009; Morgan-Fisher et al. 2013). In addition, Y27632 showed promising results in improving arthritis lesions in rodents by preventing further articular cartilage degradation (Appleton et al. 2010; Furumatsu et al. 2013).

In the context of ESC studies, Y27632 has been reported to be beneficial in several stages of ESC *in vitro* (i.e. survival/ culturing, expansion, differentiation) and *in vivo* studies. Y27632 supplemented ESC media acts as a survival factor for hESCs, by decreasing dissociation induced apoptosis (anoikis), therefore increasing cloning efficiency and scaling up ESC production for industrial use (Watanabe et al. 2007). In addition, Y27632 has been shown to enhance the post-thaw survival of embryos, hESCs, iPS, and hMSCs and facilitate differentiation (Watanabe et al. 2007; Claassen et al. 2009; Cortes et al. 2009; Gauthaman et al. 2010; Lai et al. 2010). Furthermore, the *in vivo* studies, survival of ESC-derived neural cells was enhanced by Y27632 treatment prior to and during injection in mice striatum (Koyanagi & Takahashi 2008). Moreover, Y27632 proved that ROCK is required in cell differentiation by restoring adipogenic cell differentiation in mice lacking p190-B RhoGAP (Sordella et al. 2003). ROCK dependent effects through use of Y27632, in particular in chondrogenic and osteogenic differentiation, have been reported in association with RhoA protein analysis, as will be discussed in the subsequent sections.

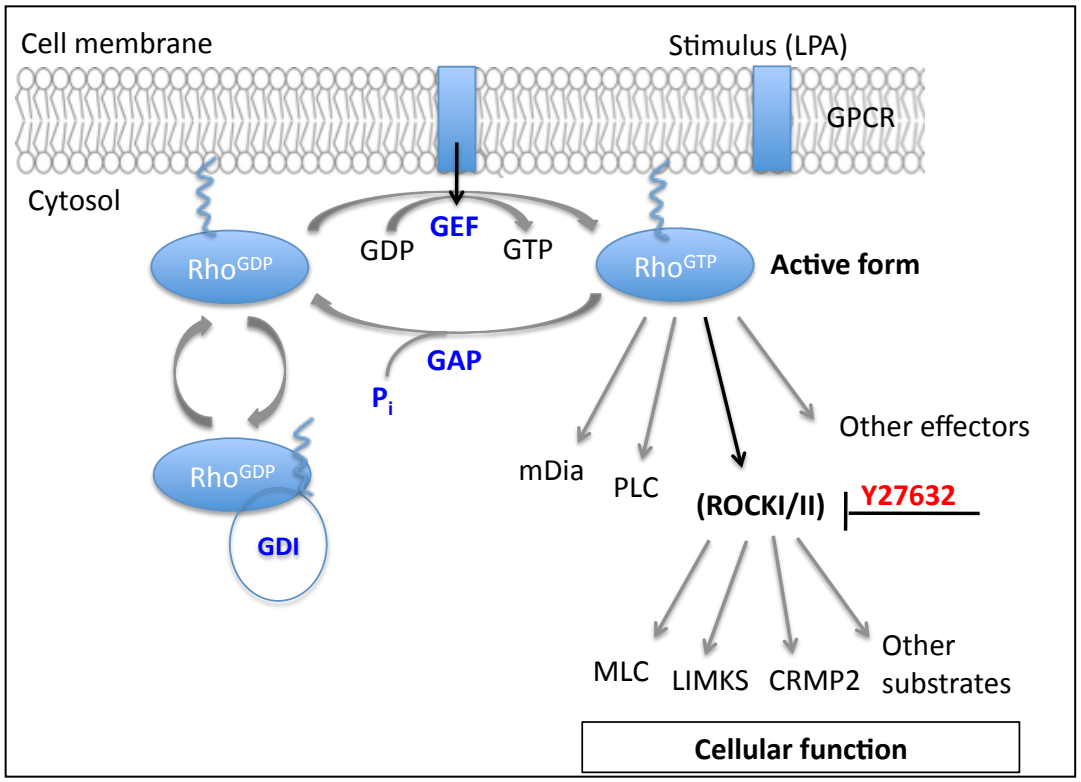


Figure 1.6: A scheme showing the Rho/ROCK GTPase signalling pathway.

The three main classes of Rho GTPase regulators (GAPs, GEFs, and GDIs), control the switch between the active and inactive states of Rho. In a resting cell, in the cytosol, Rho is present mostly in the GDP-bound form (RhoGDP) in a complex with GDI. Upon stimulation by extracellular signals such as LPA (lysophosphatidic acid) and activation of G-protein coupled receptors (GPCR), Rho is dissociated from Rho GDI (guanine-nucleotide-dissociation inhibitor) and targeted to specific membranes. At the membrane, GEFs is activated: converting RhoGDP to RhoGTP, which interacts with its specific effectors and exerts its functions. RhoA activates down stream effectors such as mammalian diaphenous (mDia), PLC, and ROCK (I/II). Once ROCK (I/II) is activated it phosphorylates downstream target substrates; MLC: myosin light chain, LIM-kinase, and collapsin response mediator protein 2 (CRMP2). Y27632 is a pharmacological inhibitor of ROCK signalling (modified from Jaffe & Hall 2005, Iden & Collard 2008 and Schofield & Bernard 2013).

1.12.3 RhoA/ROCK signalling and chondrogenesis

RhoA GTPase signalling in cartilage development has been an area of research focus due to its role in connecting signals from the extracellular matrix to the actin cytoskeleton and cellular morphology, thus it may influence other cellular activities such as cell cycle progression, gene expression, lineage commitment, differentiation, and apoptosis (Wang et al. 2004; Woods et al. 2005; Woods & Beier 2006; Kumar & Lassar 2009).

Chondrocytes display a characteristic cortical actin organization that differs from the fibrillar organisation seen in precursor cells or de-differentiated chondrocytes (Wang et al 2004). Inhibition of actin polymerisation using mycotoxin (cytochalasin B) was proved by stimulating rounding of chondrocytes in monolayer cultures, and regaining of the chondrocyte phenotype as well as expression of chondrogenic specific gene markers (Benya et al. 1988; Zanetti & Solursh 1984). In addition, the actin cytoskeleton has been shown to regulate chondrocyte differentiation in the growth plate whereby overexpression of actin-binding protein (adservin) causes rearrangement of the actin cytoskeleton, increases cell volume, and upregulates hypertrophic chondrocyte markers (Wang et al. 2004).

Furthermore, the mechanism of the actin cytoskeleton effect on chondrogenesis was linked to RhoA signalling. RhoA signalling has been proven to negatively regulate chondrogenesis. Stimulation of RhoA signalling has been shown to cause distribution of actin stress fibres in vitro and deformation of chondrocytes to a fibroblast-like phenotype (Woods et al. 2005). In addition, overexpression of RhoA inhibits chondrocyte differentiation (Wang et al. 2004; Woods et al. 2005; Woods & Beier 2006; Kumar & Lassar 2009). Furthermore, loss of RhoA expression and activity was demonstrated during dedifferentiation of chondrocytes in alginate and micromass cultures. These results were proved using dominant negative RhoA and Rho antagonist C3 transferase (Kumar & Lassar 2009).

The effect of RhoA on chondrogenesis has been linked to its downstream effector ROCK. Woods et al. (2005) showed in ATDC5 cultures that the inhibition of ROCK signalling by using ROCK inhibitor Y27632 stimulated

chondrogenesis, as was evident by the increase in glycosaminoglycan synthesis and enhancement in mRNA Sox9 expression. Meanwhile, overexpression of RhoA in ATDC5 cells showed the opposite results. These results were reproduced in micromass limb bud cultures. The stimulatory effect of RhoA/ROCK signalling inhibition on chondrogenesis is linked to cell shape and the actin cytoskeleton. It has been shown that ROCK inhibition produced spheres containing cells with chondrocyte-like morphology and exhibited reorganisation of the cortical organisation of actin filaments. The actin cytoskeleton controlled Sox9 expression whereby both cytochalasin D (binds monomeric actin and inhibits actin cytoskeleton) and colchicine (inhibitor of microtubule polymerization) were seen to reduce Sox9 expression (Woods et al. 2005).

Mechanistically, Woods et al. (2005) showed that the effect of ROCK inhibition on Sox9 expression is through stimulation of Sox9 promoter activity, thus suggesting that Sox9 is regulated at the transcriptional level. Sox9 expression is known to be required for the expression of Sox5 and 6 (Smits et al. 2001; Akiyama et al. 2002), from which all 3 genes (Sox trio) are required for induction of chondrogenesis and expression of chondrogenic specific extra cellular matrix markers such as Col II (Lefebvre et al. 2001; Ikeda et al. 2004; Han & Lefebvre 2008). Woods & Beier (2006) demonstrated that the upregulation of Sox9 expression was accompanied by upregulation in the expression of Sox5, Sox6, Col II and Acan at the mRNA level (transcript level). In addition, Sox9 expression was accompanied by an increase in the phosphorylation level of Sox9 (Woods & Beier 2006).

Several other reports demonstrated the stimulatory effect of inhibition of ROCK signalling on chondrogenesis. It was found that ROCK inhibition caused super induction of Sox9 expression in a monolayer of articular cartilage, thus rescuing the phenotype of de-differentiated chondrocytes (Tew & Hardingham 2006). Another report suggested that RhoA/ROCK signalling regulated Sox9 transcriptional activity through actin polymerization mediated by protein kinase A phosphorylation of Sox9 (Kumar & Lassar 2009). Recently, Sox9 has been demonstrated to correlate with Mef2c in down regulating Runx2 and Col X, thus preventing chondrocyte terminal differentiation to hypertrophic chondrocytes.

This may suggest that Rho GTPases may function upstream of Sox9 during chondrocyte differentiation (Dy et al. 2012).

To the contrary, other reports showed an antagonistic effect of RhoA/ROCK on chondrogenesis. This was explained by differences in cell culture (i.e. 2D versus 3D) model, cell origin, or developmental stage of cells, which might influence the effect of RhoA/ROCK signalling on chondrogenic differentiation and maturation. Woods & Beier (2006) reported that the inhibition of Rho/ROCK signalling demonstrated an increase in Sox9 expression in micromass cultures (Woods et al. 2005), which would be expected to upregulate cartilage specific extracellular markers; Col II and Acan. However, there was a decrease in Sox9 expression, which is opposite to the results seen in ATDC5 and primary cell monolayer cultures. This was unexpected since Sox9 expression is essential for the activation of the downstream markers Col II and Acan (Woods & Beier 2006). In addition, cytochalasin D down regulated the expression of Col II and Acan, confirming the ROCK inhibitor results, and jasplakinolide (inducer of actin polymerisation) was shown to recover Sox9 expression (Woods & Beier 2006).

Furthermore, inhibition of RhoA/ROCK signalling has also been shown to control the maturation of chondrocytes. Wang et al. (2004) demonstrated in both the ATDC5 cell line and in primary chondrocyte micromass cultures that overexpression of RhoA in ATDC5 cells enhanced the proliferation of chondrocytes and delayed hypertrophy as marked by decreased expression of Col X, BSP and MMP13 as well as reduction in the induction of alkaline phosphatase activity and mineralisation. In addition, ROCK inhibition via Y27632 enhanced hypertrophy of chondrocytes (Wang et al. 2004). Therefore, the negative control of RhoA/ROCK signalling on hypertrophic differentiation of chondrocytes suggests that it might negatively affect osteogenic differentiation as well. This will be further discussed in the next section.

In conclusion, the majority of studies showed an agonistic effect of ROCK signalling on chondrogenesis, although others showed an antagonistic effect. As mentioned, this was dependent on many factors, including differences in the cell culture model, cell origin, developmental stage of cells, culture condition, growth factors and cytokines, all of which might influence the effect of ROCK signalling on chondrogenic differentiation and maturation. The effect of ROCK

signalling has not yet been investigated in an ESC culture model, which will allow for a more controlled approach to tracing the signalling effect on different stages of ESC development during the differentiation process *in vitro*. In addition, given that ROCK signalling is known to regulate Sox9 expression and activity, using an ESC culture model may progress understanding of the exact mechanism of ROCK signalling in modulating chondrogenesis, thus leading to better understanding of the role of ROCK signalling in cartilage development and disease. Therefore, the next sections will focus on the use of ESCs in an approach that recapitulates embryonic developmental of cartilage and bone lineages to further understand cell specification, differentiation, and maturation.

1.12.4 ROCK signalling and osteogenesis

The influence of the ROCK pathway has also been studied in osteoblast differentiation. Interestingly, as has been reported in chondrogenic differentiation studies, controversial results were reported, defined by variations in culture conditions, such as cell origin, developmental stage of cells as well as matrix stiffness, which were shown to influence the way ROCK signalling controls osteoblast differentiation and maturation.

The Rho/ROCK pathway has been demonstrated to be a negative regulator of osteoblast differentiation. Our laboratory demonstrated that Rho/ROCK signalling inhibition increased osteogenic differentiation in mouse calvarial cells, marked by an increase in bone nodule number and mineralisation, as well as upregulation of the ALP and OC gene expression (Harmey et al. 2004). Similarly, activation of Rho by Pasteurella Multocida Toxin (PMT) led to a decrease in osteoblast differentiation and an increase in proliferation. Yoshikawa et al. (2009) also showed that ROCK inhibition using Y27632 stimulated osteogenesis both *in vivo* and *in vitro*, with upregulation of ALP and OC expression and increased nodule formation compared to the untreated cultures. The osteogenic effect of ROCK inhibition was further confirmed by means of using constitutively-active and dominant-negative ROCK constructs in ST2 cells (Yoshikawa et al. 2009). This stimulatory effect was also reproduced with a different ROCK inhibitor, Hydroxyfasudil, using MC3T3-E1 cells (Kanazawa et al. 2009) and in human primary osteoblast cultures (Ohnaka et al. 2001), therefore, confirming the anabolic effect of Rho/ROCK signalling inhibition on osteogenesis.

Mechanistically, several reports suggested a link to BMP signalling for the anabolic effect of RhoA/ROCK signalling on osteogenic differentiation. BMP (BMP2 and 4) expression was upregulated simultaneously with the increase in ALP and OC expression upon the inhibition of RhoA/ROCK signalling (Harmey et al. 2004; Yoshikawa et al. 2009). Similarly, Hydroxyfasudil upregulated BMP2 expression in MC3T3-E1 cultures (Kanazawa et al. 2009).

In support of the association between BMP signalling with RhoA/ROCK signalling several reports have proved this association by the use of other

classes of pharmacological inhibitors, that have indirect effects on Rho/ROCK, such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (Amin et al. 2013). Ohnaka et al. (2001) demonstrated that the enhancement of BMP2 and OCN mRNA expression in osteoblasts induced by hydroxyfasudil is also proved by the inhibition of the mevalonate pathway (metabolite downstream of HMG-CoA) by pitavastatin (lipophilic statins), which suppressed RhoA translocation induced by lysophosphatidic acid.

Recently, Prowsein et al. (2013) showed that RhoA/ROCK inhibition using Y27632 stimulated osteogenesis in rat calvarial osteoblasts cultured on tissue culture plastic. In addition, ROCK inhibition not only stimulated osteogenesis, but also effectively speeded up maturation and mineralisation in synergy with topography modification using SLA (sandblasted large grit, acid etched) topography, suggesting the interaction of RhoA/ROCK signalling with surface roughness in controlling differentiation and maturation.

To the contrary, Meyers et al. (2005) reported that transfecting hMSCs with an adenovirus containing constitutively active RhoA inhibited osteogenic differentiation in modelled microgravity cultures. Furthermore, Khatiala et al. (2009) demonstrated similar results to the Meyers study. However, the negative effect of RhoA/ROCK inhibition on osteoblast differentiation was also linked to ROCK and influenced by hydrogel stiffness. MC3T3-E1 cells cultured in stiff hydrogel matrices showed a reduction in the expression of Runx2, BSP, OC and ALP activity as well as osteoblast mineralisation upon inhibition of RhoA/ROCK signalling by using dominant negative RhoA, cell-permeable C3 transferase (C3) and Y27632. The reduction in Runx2 expression was accompanied by a reduction in extracellular signal-regulated kinase (ERK) activity, whereas overexpression of constitutively active RhoA showed an increase in ERK activation and stimulation of osteogenic differentiation (Khatiwala et al. 2009). Therefore, mechanistically, this suggests that ERK and Runx2 regulate the osteogenic effect of RhoA/ROCK on osteogenic differentiation. In addition, exerting external forces such as oscillatory fluid flow in C3H10T1/2 murine mesenchymal stem cell cultures resulted in activation of both RhoA and ROCK, which induced Runx2 expression. This was reduced upon application of Y27632, cytochalasin D or jasplakinolide. In parallel, the chondrogenic marker Sox9 and adipogenic marker PPARγ were negatively

regulated by RhoA/ROCK and actin cytoskeleton tension (Arnsdorf et al. 2009). Furthermore, McBeath et al. (2004) proposed that the effect of RhoA/ROCK signalling on osteogenesis may be influenced by the development stage of cells and this effect is differentially regulated by cell density and shape using a micro-pattern approach. Expressing constitutively active RhoA in un-spread cells led to osteogenic fate over adipogenic fate, particularly in the absence of osteogenic inducing growth factors or in adipogenic media. Cells infected with adenovirus encoding dominant negative RhoA cells showed an adipogenic fate, thus switching cell fate to the adipogenic lineage. Interestingly, only in spread cells was constitutively active RhoA shown to induce osteogenesis, while dominant-negative RhoA induced adipogenesis in round cells.

On the contrary, ROCK seems to control cell fate in respect to cell shape. Constitutively-active ROCK induced osteogenesis in round and spread cells, suggesting that ROCK is downstream of both cell shape and soluble factors (McBeath et al. 2004). The study identified no alteration in proliferation or apoptosis of the cells upon RhoA exposure. This study highlights that the developmental stage of cells might play a significant role in influencing the response of cells to RhoA/ROCK signalling as well as controlling cell fate. Sordella et al. (2003) confirmed the effect of RhoA on cell fate determination in the absence of soluble factors. In addition, the study proposed that RhoA might influence cell fate decision into adipocytes and myoblasts in embryonic fibroblasts derived from mice deficient in an inactivator of Rho, p190-B RhoGAP. This study, however, showed no link to cytoskeleton (Sordella et al., 2003). In summary, controversial results have been produced on the role of ROCK signalling in osteogenic differentiation, as with chondrogenic differentiation. These opposing reports suggest that studying ROCK signalling is a complex process and may be influenced by many different factors, such as species differences, cell origin, stage of cell development, as well as the involvement of extracellular matrix stiffness. In addition, ROCK signalling is a complex pathway linking external signals to the internal part of the cell, the involvement of integrin, cell cytoskeleton, cell shape, cross talk between ROCK different targets as well as the effect of upstream proteins such as RhoA and other Ras superfamily members, all of which add to the complexity of studying the potential role of ROCK in chondrogenic and osteogenic differentiation processes.

Hypothesis and aims of the study

It is now well-established that ESCs are an attractive cell source for advancing *in vitro* differentiation techniques that recapitulate the embryonic development and differentiation of specific cell types. This occurs through a directed and controlled set of experimental conditions that also allow the testing of putative novel regulators of differentiation that can eventually be translated further to clinical applications. Based on the current knowledge in the ESC differentiation field described in the Introduction, it is clear that the derivation and characteristics of the earliest embryonic chondrocyte and osteoblast precursors are not well understood. It is also clear that previous studies exploring the role of the ROCK pathway in cartilage and bone development has produced controversial findings.

Therefore, the work in this thesis will take a developmental culture approach using mESCs to (1) establish a novel system for studying the ontogeny of chondro-osteoprogenitor cells and differentiation to chondrocyte and osteoblast lineages, and (2) use this system to unravel the developmental role of ROCK signalling on chondrogenesis and osteogenesis. The specific Aims are described below:

1. To establish and optimise a step-wise differentiation system of mESCs using defined factors, through mesoderm specification, enrichment and commitment to cartilage and bone cell lineages, and differentiation to maturity *in vitro*. This will test the hypothesis that defined combinations and exposure to specific developmental factors can produce the optimal mesodermal subpopulations for chondro-osteoprogenitor cell differentiation.

2. To investigate the role of Rho/ROCK signalling through inhibition of ROCK activity at defined stages of lineage development. This will test the hypothesis that ROCK inhibition will have differential effects on chondrogenesis and osteogenesis depend on the stage of development and differentiation of early stem/progenitor cells versus lineage committed cells.

3. To investigate the potential of ESC-derived chondrocytes and osteoblasts generated *in vitro* to differentiate *in vivo* following xenograft transplantation. This will test the hypothesis that *in vitro*-generated chondrocytes and

osteoblasts using the protocols of Aims 1 and 2 can recapitulate developmental chondrogenesis and ossification to form functional tissue *in vivo*.

I anticipate that after achieving these Aims, that novel insights will be gained into the mechanism of cartilage and bone development that may contribute to the improvement of regenerative therapies and the treatment of cartilage and bone disorders.

Chapter 2 Materials and methods

List of reagents and abbreviations

Alcian blue	AB
Alkaline phosphatase	ALP
Bone morphogenic protein 4	BMP4
Bovine Serum Albumin	BSA
B27 supplement serum-free supplement rat hippocampal and cortical neurons	B27
Dexamethasone	Dex
1,25-dihydroxyvitamin D_3	Vitamin D_3
Distrene plasticiser xylene	DPX
Dulbecco's Modified Eagle Medium	DMEM
Ethylenediaminetetraacetic acid	EDTA
Fibroblast growth factor 2 (basic FGF)	FGF2
Foetal calf serum	FCS
β-glycerophosphate	β-GP
Growth/differentiation factor 5	GDF5
Hydrochloric acid	HCL
Iscove's Modified Dulbecco's Media	IMDM
L-Ascorbic acid	AA
leukaemia inhibitory factor	LIF
L-Glutamine	L-Glut
Magnesium chloride	MgCl

List of reagents and abbreviations

Moloney murine leukemia virus	MMLV-RT
Monothioglycerol	MTG
Chemically defined, serum-free supplement, based on N-1 formula (Growth of a rat neuroblastoma cell line in serum-free supplemented medium)	N2-Supplement
Paraformaldehyde	PFA
Phosphate-Buffered Saline	PBS
Rho-associated protein kinase (ROCK inhibitor) ($C_{14}H_{21}N_3O.2HCl$). Chemical name: *trans*-4-[(1*R*)-1-Aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide dihydrochloride	Y27632
Smoothened (Smo) agonist (Chlorobenzothiophene-containing Hh pathway)	SAG
An animal origin-free, recombinant enzyme replacment of porcine trypsin	TrypLE™
Wingless-type MMTV Integration Site Family (Member 3a)	Wnt3a

2.1 Cell culture

2.1.1 Mouse Embryonic Stem Cell (mESC) culture

As described in the introduction, mESCs are a useful system to study osteoblasts and chondroblasts from early lineage specification and commitment to differentiation stages. For all experiments, the CCE cell line (feeder-free cell line) was used (Robertson et al. 1986). Cells were differentiated using a modification of a three-phase protocol that has been published previously (Craft et al., 2013; Gouon-Evans et al. 2006) with some modification for this project as described in chapter 3.

2.1.2 mESC growth and maintenance

Cells were maintained in DMEM media supplemented with 20% batch-tested FCS (Summit), leukaemia inhibitory factor (LIF) (10^3 u/ul) (Chemicon) and $1.5x10^{-4}$ M of monothioglycerol (MTG, Sigma-Aldrich) (designated DMEM-ES media). Cells were plated in a 6-well plate (BD Falcon) coated with 0.1% gelatine (in 1% BSA/PBS). Cells were passaged when they reached near confluence (80%) and the medium was changed every other day.

To freeze down ESCs, cells were spun down at 1200 rpm for 5 minutes at 4°C and re-suspended in cold freezing medium (prepared early and kept on ice) at approximately $5x10^6$ cells/vial. Cell suspensions were slowly and gently pipetted into ice-cold cryovials (1 or 1.8 ml size) (labelled and stored at -80°C prior to freezing). Vials were frozen at -80°C and then transferred to liquid nitrogen for long-term storage.

2.1.3 mESC differentiation via generation of Embryoid Bodies (EBs)

To prepare ESCs for differentiation, they were passaged once more in IMDM-ES+LIF media where DMEM was replaced by IMDM in the medium followed by plating of ESCs in SFD medium to start the differentiation process. This stage of differentiation is identified as (0d), which is the start of differentiation (Fig.2.1) and will be discussed in details in next section 2.1.3.1.

2.1.3.1 Mesoderm induction (phase I: 2-4d)

At 0d of differentiation (start of differentiation), mESCs were trypsinised (0.25% trypsin/EDTA, Sigma-Aldrich) and cultured in 60mm dishes (Sterilin) at $7.5x10^6$ cell/dish in SFD medium. This stage allowed mESCs to form embryoid bodies (EBs). The SFD medium used in the differentiation experiment consisted of N2-Supplement and B27 (without retinoic acid) (Invitrogen), 10% BSA/PBS (Sigma-Aldrich), IMDM (Sigma-Aldrich), F12 (Invitrogen), 1% L-glutamine and 1% Penicillin/Streptomycin (Sigma-Aldrich). SFD medium was then supplemented with 50ug/ml L-ascorbic acid (AA), L-glutamine (5mM) and MTG (3ul/1ml) (Sigma-Aldrich) just prior to adding the medium to the cells. This medium will be referred to as SFD+supplements (SFD+S). After 2d of differentiation, EBs formed were harvested and dissociated with 0.25% trypsin/EDTA then re-aggregated in SFD+S consisting of mesoderm-inducing factors: 9 ng/ml Activin A (cat. no. 338AC), 25ng/ml Wn3a (cat. no. 1324WN), and 100 ng/ml Noggin (cat. no. 1967NG) (R&D) cultured at the same initial cell density for 2 days (Fig. 2.1). A one-day mesoderm induction was also tested as shown in next section.

2.1.4 One day mesoderm induction (phase I: 2-3d)

The mesoderm induction medium used for a 2-day mesoderm induction (from 2-4d) discussed in the previous section 2.1.3.1, was also used in testing a 1-day mesoderm induction period (from 2-3d) for both chondrogenic and osteogenic differentiation in phase III.

2.1.4.1 Mesoderm enrichment (phase II: 4-7d)

At 4d of differentiation (following a 2-day mesoderm induction), formed EBs were dissociated in TrypLE and re-aggregated in a 24-well plate (ultra low attachment) (Costar) at $0.25x10^6$ cell/well in a suspension culture. At this time only FGF2 (10ng/ml) (cat. no. 234-FSE) (R&D) was added to SFD+S medium for mesoderm enrichment (Fig. 2.1).

2.1.4.2 Monolayer differentiation (phase III: 7-21d)

At 7d (following 3 days of re-aggregation), re-aggregates were dissociated with TrypLE and plated as single cells in a 0.1 % gelatinised, 96-well plate (BD Falcon) at a cell density of (10,000 cell/well) in a monolayer culture. The cells were cultured in SFD+S consisting of either chondrogenic or osteogenic differentiation media as will be described in the next sections. All cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. The medium was changed at 2-3 day intervals (Fig. 2.1).

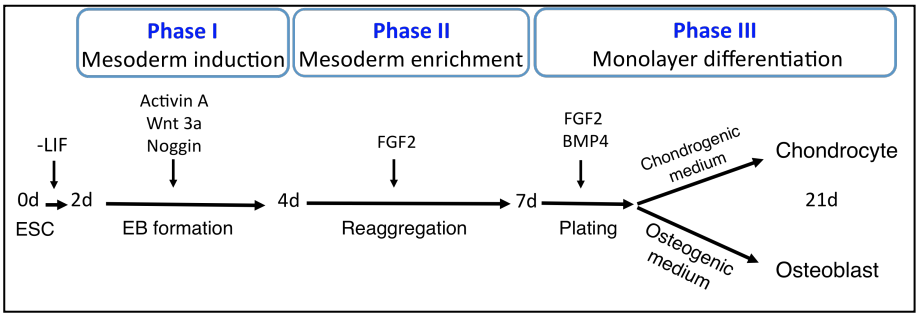


Figure 2.1: Step-wise, serum free directed ESC/EB differentiation culture model.

The start of the differentiation was marked once CCE mESCs had been cultured in the absence of LIF for 2 days. At 2d of differentiation, ESCs were cultured in SFD+S consisting of mesoderm-inducing factors: 9 ng/ml Activin A, 25ng/ml Wnt-3a, and 100 ng/ml Noggin for 2 days (phase I: from 2-4d). At 4d of differentiation, formed EBs were cultured in FGF2 (10ng/ml) for 3days (phase II: from 4-7d). At 7d of differentiation, aggregates were plated as single cells in a monolayer culture containing differentiation media for 2 weeks (phase III: 7-21d). Chondrogenic media details are given in section 2.1.5 and osteogenic media details in section 2.1.6.

2.1.5 Chondrogenic differentiation

For chondrogenic differentiation, single cells were cultured in SFD+S media consisting of FGF2 (10ng/ml) and recombinant human BMP4 (100ng/ml) (R&D) in addition to ascorbic acid and L-glutamine (Table 2.1).

Reagent	Final Conc.	Company/cat. no.
SFD+S (A.A+L-G+MTG)	1ml/ml	-
Recombinant Human FGF2	10ng/ml	R&D/234-FSE
Recombinant Human BMP4	100ng/ml	R&D/314BP

Table 2.1**:** Chondrogenic differentiation media.

2.1.6 Osteogenic differentiation

For osteogenic differentiation, single cells were cultured in SFD+S media consisting of FGF2 (10ng/ml), BMP4 (100ng/ml), ascorbic acid, L-glutamine, in addition to, Dexamethasone (10-7M) (Sigma-Aldrich) and β-GP (10uM) (Sigma-Aldrich) (Table 2.2).

Reagent	Final Conc.	Company/cat. no.
SFD+S (A.A+L-G+MTG)	1ml/ml	-
Recombinant Human FGF2	10ng/ml	R&D/234-FSE
Recombinant Human BMP4	100ng/ml	R&D/314BP
Dexamethasone	10^{-7} M	Sigma-Aldrich/D8893
β-glycerophosphate	10uM	Sigma-Aldrich

Table 2.2: Osteogenic differentiation media.

2.2 Analysis of ROCK inhibition via Y27632 on mESC differentiation

At 4d (following 2 days in mesoderm induction media), formed EBs were dissociated and re-aggregated in a 24-well plate (ultra low attachment) at $0.25x10^6$ cells/well. At this time Y27632 (10uM) (Merck) was added to SFD+S media in the presence of FGF2 (Fig. 2.4). At 7d (after 3 days of re-aggregation), cells from Y27632 pre-treated and not pre-treated at phase II, were plated in a 0.1 % gelatinised, 96-well plates at a cell density of 10,000 cells/well. The cells were cultured in the presence and absence of Y27632 in either chondrogenic (table 2.1) or osteogenic media (table 2.2) for 2 weeks (Fig. 2.2).

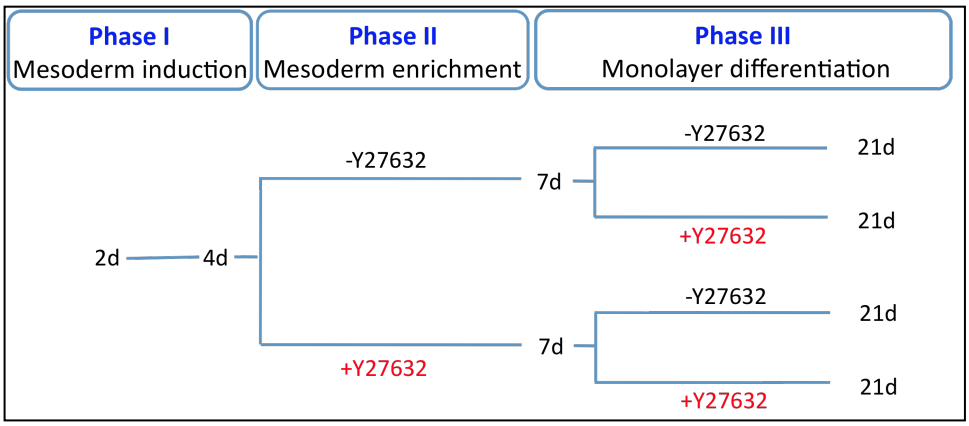


Figure 2.2**:** Step-wise, serum free directed ESC/EB differentiation culture model showing stage specific addition of Y27632**.**

A schematic diagram showing 3-stage differentiation phases described in (figure 2.1). At 4d of differentiation (phase II), formed EBs were cultured in FGF2 (10ng/ml) for 3days in the presence and absence of Y27632 (10uM). At 7d of differentiation (phase III), cells from Y27632 pre-treated and not pre-treated at phase II, were plated as single cells in a monolayer culture in the presence and absence of Y27632 in either chondrogenic (table 2.1) or osteogenic (table 2.2) media for 2 weeks.

2.3 Modification of the differentiation phase (phase III) protocol

The culture medium used in the differentiation phase (phase III: 7-21d) was modified as presented in (chapter 5) and (chapter 7, section B) to investigate the *in vitro* differentiation and maturation potential of chondrogenic and osteogenic cells, respectively.

2.3.1 Culturing cells in FGF2 either alone or in combination with BMP4 in the differentiation phase (phase III)

At 4d of differentiation, EBs were cultured in SDF+S containing FGF2 and Y27632 for 3-days of the reaggregation period. At 7d of differentiation, aggregates were plated in a 0.1 % gelatinised, 96-well plate at a cell density of 10,000 cells/well in a monolayer culture in SFD+S in the presence of Y27632 with either FGF2+BMP4, FGF2 alone or BMP4 alone for 3 weeks (Fig. 2.3A). This was also tested in osteogenic media, which consisted of SFD+S in the presence of Y27632 with either FGF2+BMP4, FGF2 alone or BMP4 alone, in addition to, Dexamethasone and β-GP (Fig. 2.3B).

2.3.2 Primary osteoblast cultures

Primary calvarial osteoblasts were isolated as described by Harmey et al. (2004) and cultured in differentiation media, which consisted of AA and β-GP, in both the presence and absence of batch-tested serum. The differentiation media was then supplemented with FGF2 or a combination of FGF2 and BMP4 for 2 weeks to test the effect of FGF2 on cells other than ESCs.

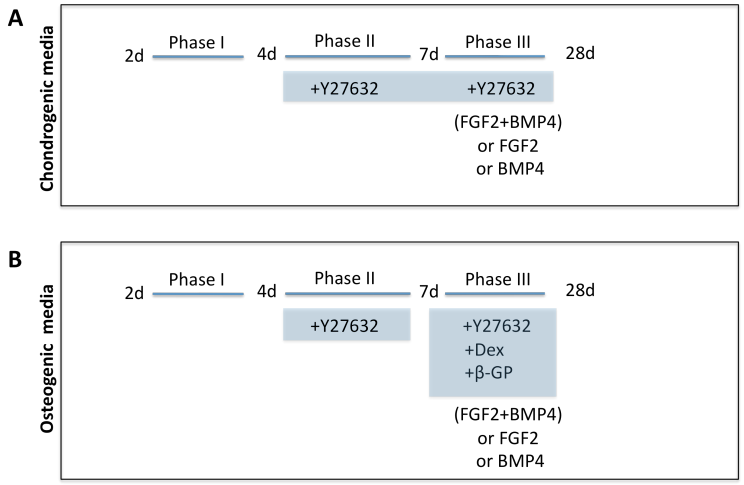


Figure 2.3**:** A schematic diagram showing the addition of FGF2 alone or in combination with BMP4 in chondrogenic or osteogenic media.

A) A differentiation scheme showing the timing of factors additional to the culture media in phases II and III. In phase II, cells were cultured in FGF2 and Y27632. Then in phase III, cells were cultured in chondrogenic media containing Y27632 in the presence of FGF2 and BMP4 either alone or in combination for 3 weeks. B) A differentiation scheme showing the timing of factors additional to the culture media in phase II and III. In phase II, cells were cultured in FGF2 and Y27632. Then in phase III, cells were cultured in osteogenic media containing Dexamethasone, β-GP and Y27632 in the presence of FGF2 and BMP4 either alone or in combination for 3 weeks.

2.3.3 Culturing cells in temporal FGF2 in the differentiation phase (phase III)

For testing chondrogenic differentiation in Y27632 and temporal FGF2 containing media, cells from Y27632 pre-treated and not pre-treated at phase II were plated on 7d in SDF+S consisting of FGF2 and BMP4 (100ng/ml) in the presence of Y27632. After one week of culture, FGF2 was removed from the media and cells were cultured for another week in BMP4 and Y27632 for chondrogenic differentiation, or further supplemented with Dexamethasone and β-GP for osteogenic differentiation. The temporal FGF2 protocol will be referred to as (Temporal FGF culture) later on as discussed in chapter 5 (Fig. 2.4).

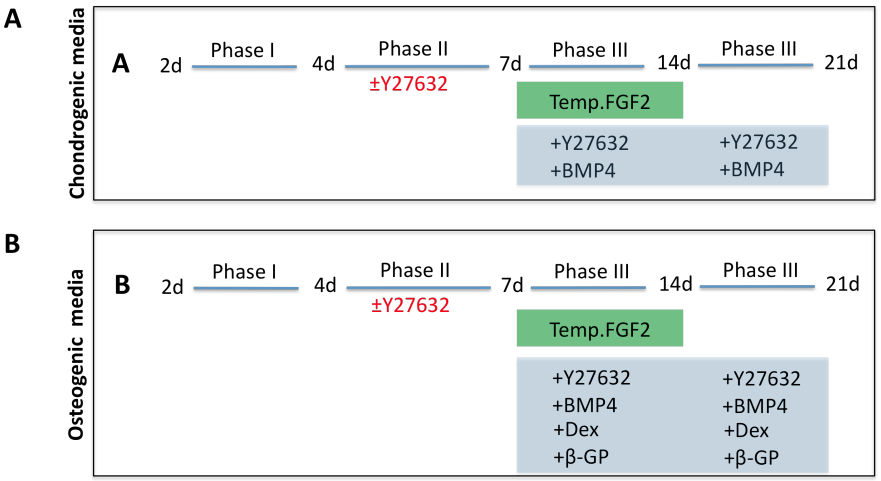


Figure 2.4**:** Culturing cells in temporal FGF2 in the differentiation phase (phase III).

A differentiation scheme showing the timing of factors additional to the culture media in phases II and III. In phase II, cells were cultured in FGF2 in the presence and absence of Y27632. Then in phase III, cells were cultured in chondrogenic media containing Y27632, FGF2 and BMP4. At 14d, FGF2 was removed from the culture media and cells were cultured in media containing Y27632 and BMP4 for another week. B) A differentiation scheme showing the timing of factors additional to the culture media in phases II and III. In phase II, cells were cultured in FGF2 in the presence and absence of Y27632. Then in phase III, cells were cultured in osteogenic media containing Y27632, FGF2, BMP4, Dexamethasone and β-GP. At 14d, FGF2 was removed from the culture media and cells were cultured in media containing Y27632 and BMP4 for another week.

2.3.4 Culturing cells in temporal FGF2 and GDF5 in the differentiation phase (phase III)

For testing chondrogenic differentiation in media containing GDF5, cells from Y27632 pre-treated and not pre-treated at phase II, were plated at 7d in SDF+S consisting of FGF2 and GDF5 (30ug/ml) (R &D) in the presence of Y27632. After one week of culture, FGF2 was removed from the media and cells were cultured for another week. This culture media will be referred to as (Temporal FGF: GDF5 culture) later on (Fig. 2.5).

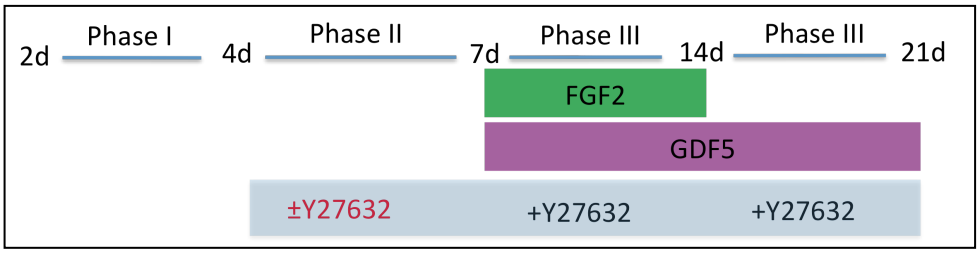


Figure 2.5: A schematic diagram showing addition of temporal FGF2 and GDF5 in phase III.

A differentiation scheme showing the timing of factors additional to the culture media in phases II and III. In phase II, cells were cultured in FGF2 in the presence and absence of Y27632. Then in phase III, cells were cultured in FGF2 and GDF5 in the presence of Y27632. FGF2 was removed from the culture media and cells were cultured in media containing Y27632 and GDF5 for another week.

2.3.5 Prolonging the culture period to test mineralisation in the differentiation phase (phase III)

The original standard culture period used for osteogenic differentiation in phase III was 2 weeks (from 7d to 21d). The culture period was then prolonged for another 2 weeks (i.e. from 21d to 35d). Cells were treated with Y27632 in phase II, and subsequently, in phase III, cells were cultured in osteogenic media (table 2.2) in addition to Y27632 for 35 days.

2.3.6 Culturing cells in osteogenic inducing agents in the differentiation phase (phase III)

Cells from Y27632 pre-treated and not pre-treated in phase II were cultured in media in phase III in osteogenic media (table 2.2) containing Y27632, in addition to one of the following factors; SAG, Wnt3a, VitD3 or β-GP (2mM) for 3 weeks. Further details are in chapter 6 (Fig. 2.6).

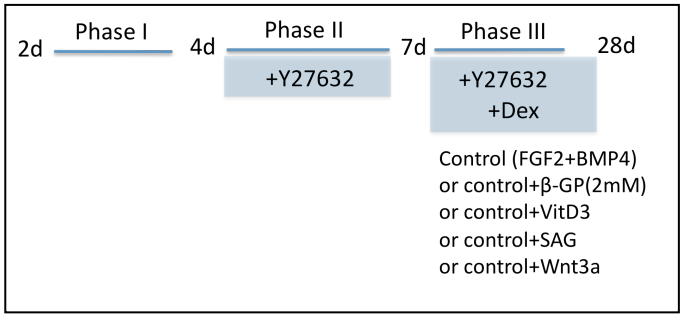


Figure 2.6**:** Culturing cells in Y27632 with known osteogenic inducing factors.

A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. In phase III, cells were cultured in osteogenic media supplemented with one of the following osteogenic factors: β-GP (2mM), VitD3 (10^{-8}M), SAG (100nM) and Wnt3a (10ng/ml) for 3 weeks.

2.3.7 Culturing cells in differentiation media containing serum

Cells were cultured in phase II in the presence of Y27632, and in phase III cells were cultured in osteogenic media (Table 2.1) containing Y27632 for 28d, and additionally batch-tested FCS was added at three different concentrations (10%, 1%, and 0.1%).

2.3.8 Re-plating differentiated cells in phase IV (21-28d)

Cells Y27632 pre-treated and not pre-treated in phase II were cultured in osteogenic media in presence of Y27632 in phase III, for 2 weeks. FGF2 was added only for one week (7-14d) and removed afterwards. Cells were then dissociated using TrypLE and replated in 0.1 % gelatinised, 48-well plates (Corning® Costar®) in osteogenic media containing BMP4, Dexamethasone and β-GP in the presence of Y27632 (Fig. 2.7).

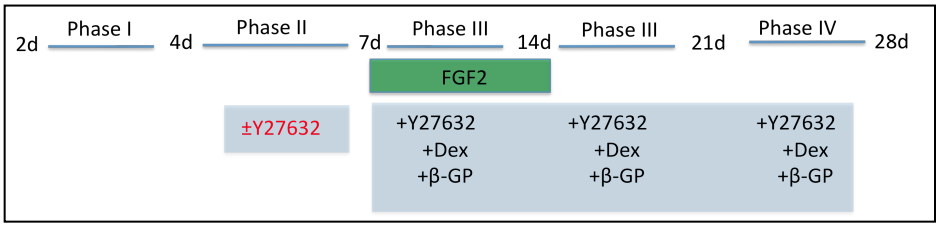


Figure 2.7:A schematic diagram showing ESC/EB differentiation culture model with the re-plating phase (phase IV).

A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. Cells from Y27632 pre-treated and not pre-treated in phase II, were cultured in phase III, in osteogenic media containing BMP4, Y27632, Dex, and β-GP. At 14d, FGF2 was removed from the culture media and cells were cultured in Y27632 BMP4, Dex, and β-GP for another week. At 21d, the cells were re-plated and cultured in media containing BMP4, Dex, and β-GP in the presence and absence of Y27632 for 1 week.

2.4 Kidney capsule *in vivo* transplantation

At the mesoderm enrichment phase (phase II), cells were cultured in FGF2 in the presence and absence of Y27632 and then harvested at 7d for transplantation under the kidney capsule of adult immunocompromised (SCID) mice, in the form of aggregates or as a single cell suspension in matrigel. Matrigel was then transplanted without cells as a control (Fig. 2.8A).

At the differentiation phase (phase III), cells from Y27632 pre-treated or not pre-treated were the cultured in phase III in osteogenic media in the presence of Y27632. At 21d of differentiation, monolayers were harvested by mechanically scraping the monolayer from the well plate as one piece and surgically placing it under the kidney capsule of adult immunocompromised (SCID) mice (Fig. 2.8B). After that, the kidneys were surgically removed and fixed in 4% paraformaldehyde overnight, and then decalcified for 1 day. Kidney samples were then processed and embedded in wax for histology 2.5.5.1. Transplantations were performed in 2 independent experiments. All animal studies had been reviewed and approved by the Institutional Animal Care and Use Committee at King's College University in accordance with the policy regarding the use and care of laboratory animals and all procedures were performed according to Home Office guidelines.

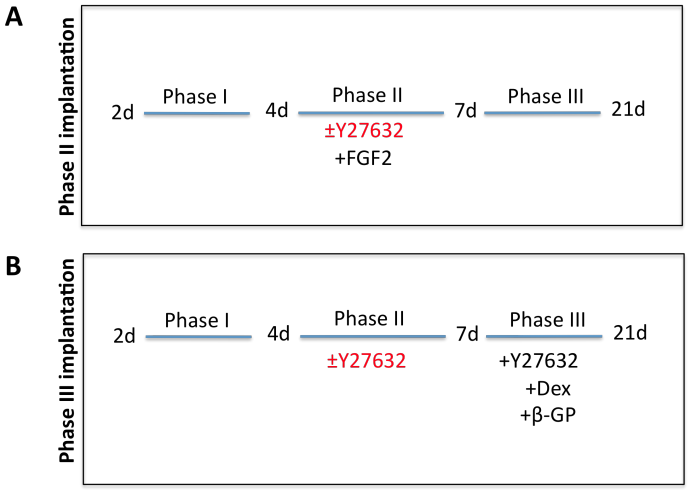


Figure 2.8: A schematic diagram showing the two time points of in vivo transplantation.

A differentiation scheme showing the timing at which Y27632 was added. In phase II, cells were cultured in FGF2 in the presence and absence of Y27632. Then after 3 days in culture (at 7d), cells were harvested and transplanted under the kidney capsule of adult immunocompromised (SCID) mice, in the form of aggregates or as single cells in matrigel suspension, and harvested after 3 weeks. B) A differentiation scheme showing the timing at which Y27632 was added. In phase II, cells were cultured in FGF2 in the presence and absence of Y27632. In phase III, cells were cultured in the presence and absence of Y27632 in osteogenic media. Then, after 2 weeks in culture (at 21d), cells were harvested and transplanted under the kidney capsule of adult immunocompromised mice (SCID), in the form of a monolayer, and harvested after 3 weeks.

2.5 Data analysis

2.5.1 Histochemical staining:

The cultured cells were fixed at 21d with 4% paraformaldehyde (PFA) in PBS for 15 minutes and washed with distilled water prior to staining to remove any residual fixing material.

2.5.2 Alkaline phosphatase staining and quantification

Alkaline phosphatase (ALP) activity of osteoblasts was examined using a salt solution prepared from a mixture of 5mg Naphthol AS MX-PO4, 200µl N,N-dimethylformamide (DMF) added to 25ml Tris-HCL (0.2M, pH8.3), 25ml distilled water and 30mg Fast Red TR . All staining reagents were purchased from Sigma-Aldrich (Dorset, UK). The solution was filtered through a 0.45um filter (NALGENE 190-2545) and immediately added to fixed cells and left to incubate at room temperature until the cells turned red (working time 10 minutes until solution start to precipitate). Following incubation, the cells were rinsed with distilled H_2O and visualised under microscope (Olympus). Plates were also scanned using an Epson Perfection 1200 scanner (Herts., UK). Quantification of the stained area was done using Image J ® software to quantifying the threshold area.

2.5.3 Alcian Blue staining and quantification

To stain acidic proteoglycans in chondrocyte nodule monolayers, a 1% Alcian blue solution prepared in 0.1N HCl was used. The cells were washed for 3x 3 minutes each with 0.1 N HCl and then stained with Alcian Blue for 8 hours or left overnight. Monolayers were then washed again with 0.1 N HCL for 3x 3 minutes per wash followed by a distilled water wash. The stained wells were prepared for quantification of staining area as described above for alkaline phosphatase staining images, using Image J ® software. When the chondrocyte nodules had coalesced, grid count method, a modification of the point-counting method was used (Grigoriadis et al 1996).

The Alcian Blue-positive area/nodules which coincided with a grid intersect was scored and expressed as a percentage of the total number of intersects.

For quantification of Alcian Blue staining, which represents GAG synthesis, Alcian Blue-stained monolayers were first solubilised using a modification of the Mushtag et al (2002) protocol as the follows; after washing the monolayer with distilled water , the wells were treated with 200ul of 6M of guanidine HCL to extract alcian blue colour for 6 hours on a rotating platform at room temperature, repeated pipetting of guanidine solution to aid in mechanically extracting alcian blue colour from 3-D chondrocyte nodules. Then extracted dye solution was transferred from each well to a labelled clean well to allow for accurate measurements using a plate reader (Thermo, Multiskan Ascent). Absorbance was measured at OD of 630nm. The quantifications were then normalised to guanidine HCL.

2.5.4 Immunocytochemistry

To analyse the expression of cartilage markers (Sox9, Col II, Col X) and bone markers (Col 1) during ESC differentiation, an indirect immunofluorescence technique was used. The monolayer was stained either *in situ* or on a slide following histological sectioning. Each antigen required specific treatment before antibody staining. Briefly, the monolayer was fixed in 4% PFA in PBS for 15 minutes and washed with distilled water and then treated according to each protein as summarised in Tables 2.3 and 2.4. After that, the monolayer was washed 3x with 5-10% gout serum blocking solution at room temperature. Primary antibody was diluted in blocking buffer then added at the optimised dilutions (Table 2.4) and incubated at 4°C overnight. The monolayer was washed with PBS 3x and incubated in secondary antibody for 45 minutes or 1 hour at room temperature in the dark. Finally, the monolayer was then incubated with 4', 6-diamidino-2-phenylindole (DAPI) (1ug/ml) or Hoechst 33342 (20ug/ml) (Sigma) for 15 min for nuclear staining. To detect Sox9 expression, staining was performed on 7d cells on following cytospinning of the cell suspension, or on monolayers from 10d cultures (ie. 3days after plating). Col I and Col II stainings were performed on 21d monolayers. Col X staining was performed on 21d monolayers following paraffin histology.

The stained monolayers were visualised using a Zeiss microscope (Axiovert200)/software Axiovision 4.8 software, or a Nikon microscope Eclipse 80i. DAPI, FITC and TRITC fluorescence were visualised with DAPI filter (excitation 358nm and emission 461 nm), FITC filer (excitation 494 and emission 518 nm) and TRIT filter (excitation 545 and emission 563) respectively.

2.5.5 Cytospin

At 7d of differentiation aggregates were dissociated and single cells were prepared for cytospin (Shandon Cytospin 3). The cells were centrifuged at 1350 rpm for 5 min and slides were fixed with 4% PFA for 15 minutes at room temperature followed by 0.2% washed with Triton-X100 for 15 min again at room temperature. Then the samples were blocked in 5% goat serum in PBS overnight at 4°C as described above prior to immunostaining, and were counterstained with 10 ug/ml Hoechst for 5-10 minutes at room temperature. The stained cells slides were visualised using Nikon microscopy.

Procedure	Reagents	Concentration	Company/ cat. no.
Pepsin digestion solution	Pepsin	10 mg/ml	Sigma-Aldrich/P-7000
	Hydrochloric acid	0.02 N	
Protein kinase digestion	10mg/ml (stock)	2ug/ml	Sigma-Aldrich/P1609

Table 2.3: Reagents used for Collagen type II immunofluorescence staining.

Table 2.4: Immunocytochemistry and pre-treatment reagents.

Antibody	Host/ Antibody type	Primary antibody		Secondary antibody		Treatments/permeabalsation	Blocking solution
		Working conc.	Company/ cat.no.	Working conc.	Company/ cat.no.		
Sox9	Rabbit polyclonal	1:200	Millipore cat no. AB5535	1:500	Goat anti-rabbit 568 Alexa Fluor Invitrogen cat no. A11011	1% triton x-100/ H_2 o for 5-15 minutes then rinsed with distilled water.	10% goat serum in PBS
Col II*	Mouse monoclonal	1:200	Millipore MAB8887	1:250	Goat anti-mouse 488 Alexa Fluor Invitrogen cat no. A11001	Pepsin digestion solution for 2-5 minutes	5% goat serum in TBS
Col X	Mouse monoclonal	1:10 from neat	(from Klaus von der Mark lab)	1:250	Goat anti-mouse Alexa Fluor Invitrogen cat	Protein kinase for 30 minutes (tissue section)	10% goat serum in PBS
Col 1	Mouse monoclonal	1:200	Abcam ab90395	1:250	Goat anti-mouse 568 Alexa Fluor Invitrogen cat	0.25%triton-100/PBS for 10-15 minutes	10% goat serum in PBS

2.5.5.1 Tissue processing and histology

The two-week (21d) differentiated monolayers were mechanically scraped under a dissecting microscope using a sterile needle to detach the edges of the monolayer from the well prior to fixation. All samples, including cell monolayers and kidney tissues were fixed in 4% paraformaldehyde, decalcified in 0.5M EDTA where appropriate, dehydrated in increasing concentrations of ethanol, then washed with histoclear^{TM}, before being embedded in paraffin and cut at 5um using microtome (leica, RM2245). Monolayers were processed for histology according to the step-wise protocol in Table 2.5. All sections were mounted on poly-lysine coated slides (super frost plus, VWR, 631-0449) then de-paraffinised and rehydrated before stained with H&E and Alcian Blue, or processed for immunocytochemistry as indicated.

Haematoxylin & Eosin (H&E) staining was used together with Alcian Blue staining to stain the nuclei (purple/blue colour) with Haematoxylin (Solmedia HST003) and eosin to stain (Solmedia HST101) cytoplasm of the cell (pink/red colour) while Alcian blue to stain chondrcocyte extracellular matrix. Sections were then de-paraffinized with Histo-clearTM 10 minute x2 and rehydrated through a series of ethanol washes (100%, 90%, 70% and 50%) 2 minutes/wash. Sections were then washed for ten minutes in distalled H_2O followed by submerging sections in Erhlich's Haematoxylin for 10 minutes. Samples were then washed for 10 minutes under running water to remove excess haematoxylin. Then sections submerged in acid alcohol for 15 seconds. Subsequently, the sections were stained with 0.5% aqueous Eosin for two minutes, then washed in distalled H_2O and dehydrated through a series of two minute ethanol washes (70%, 90% and 2x 100%). Sections were then air-dried up to one hour. After that, slides were mounted using DePex (Solmedia REA201) mounting media. Modification of timing was done for monolayer tissue sections. Sections were mounted in either aqueous or DePeX mounting media. Finally, sections were visualized under Zeizz microscope Axioskop 2 plus/ soft ware and pictures taken using AxioCamHRcamera.

Stations	Solution	Procedure
1	PBS	Wash 3x
2	70%ethanol	Incubate 15 minutes
3	90% ethanol	Incubate 15 minutes
4	100% ethanol	Incubate 15 minutes 3x
5	histoclear	Incubate 15 minutes
6	Ultraplast wax	Incubate 15 minutes 3x

Table 2.5: Monolayer tissue processing protocol.

2.5.6 Molecular analysis: Polymerase Chain Reaction (PCR)

Determination of cellular gene expression was done using reverse transcription-PCR (RT-PCR) at specific time-points during differentiation (Fig. 2.9). A two-step protocol was used. First, RNA was reverse transcribed to cDNA. Second, diluted cDNA was amplified by either quantitative or semi-quantitative RT-PCR as described below. All steps up to PCR reaction were carried out using RNase and DNase free pipette filter tips; all bench areas were cleaned using an RNase and DNase free solution (Sigma-Aldrich). The RNA and DNA work were performed on separate days to avoid cross contamination between samples.

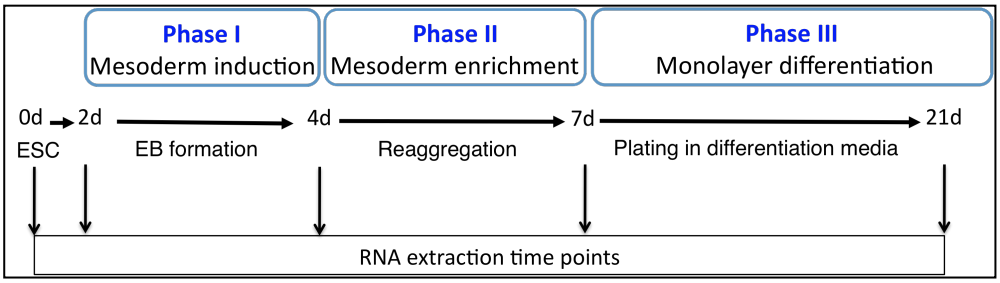


Figure 2.9: Time points at which RNA was extracted and used for PCR and gene expression analysis.

2.5.6.1 RNA extraction and quantification

RNA preparation and extraction was performed using the Qiagen RNEasy mini and micro kits following manufacturer's instructions. Briefly, cells at the indicated times were removed and dissociated using either 0.25% trypsin in EDTA (for cells from DMEM and IMDM cultures) or TrypLE (from 0d-28d) for not more than 5 minutes, then lysed by addition of appropriate volume of RLT lysis buffer containing β-mercaptoethanol (β-ME) (10ul/ml) to allow for complete release of RNA. Mechanical pipetting was performed until complete lysis of monolayer was achieved. The Lysates were stored at -80°C until RNA extraction.

The Lysates were homogenised using a QIAshredder spin column (Qiagen) to reduce the viscosity of the lysates and ensure binding of RNA to the silica membrane. RNA was isolated from lysate using a RNeasy column purification kit (Qiagen) according to the manufacturer's instructions. A Minikit (74104,74106) or Microkit (74004) was used according to cell numbers. The Minikit was used to extract RNA from 0d to 7d while the Microkit was used for RNA extraction from 14d and 21d cultures. Poly-A RNA was added to lysate before homogenising step, as an RNA carrier (4 ng/µl). DNase (nonspecific endonuclease) was included in the extraction process to that degrades DNA. The concentration of RNA was determined by measuring absorbance at 260nm (A260) in a spectrophotometer (NanoDrop 2000C, Thermo), which included measuring purity of RNA (260/280 and 260/230 ratios).

2.5.6.2 Reverse transcription (RT)

Reverse transcription was performed in two steps. First, to melt secondary structure of RNA, 1ug of RNA was mixed with random primers (Table 2.6) and denatured at 70°C for 5 minutes, then placed on ice for five minutes until proceeding with the reaction mix for cDNA synthesis (Table 2.7). The reaction mix contained MMLV-RT Reverse Transcriptase as an RNA-dependent DNA polymerase (Table 2.7). The reverse transcription reaction mix was then added to the RNA and random primer mix, and was incubated at room temperature for 10 minutes then in a hot block at 42°C for 1 hour. Then placing samples on ice stopped the reaction. Negative control samples were prepared by omitting

MMLV-RT in the reaction mix. Then cDNA was diluted 1:5 in nuclease free water and stored at 4°C for short-term use or at -20°C for long-term storage.

Reagent	Volume/reaction (ul)	Company/cat.no.
RNA	Up to 12 ul	-
Random primer 20ug	2	Promega/C118A
RNase-free water	Prepared up to 14	-
Total volume	14	-

Table 2.6: Reverse transcription (RT) reaction mix for melting of secondary structures.

Reaction mix reagents	Concentration	RT+ mix x1	RT- mix x1	Company/cat.no
dNTPs	1mM	1.25ul	1.25ul	Promega/C114G
buffer	5x	5ul	5ul	Promega/M531A
MMLV-RT	200u/ul	1ul	-	Promega/M170A
Nuclease-free H2O	-	3.75ul	(4.75)	Promega
Total volume		11ul	11ul	-

Table 2.7: Reverse transcription (RT) reaction mix for cDNA synthesis.

2.5.6.3 Primer designing, efficiency and specificity testing

Forward and reverse primers were designed using Primerblast-NCBI with exon-exon junction spanning to limit amplification to mRNA only. Some primers were found in published papers and checked using NCBI Primer-Blast for specificity (Table 2.11). Serial 1:5 dilutions of RNA expressing the gene of interest (E11.5-14.5 for chondrogenic genes, primary osteoblasts (POB) for osteogenic genes) were used to run standard curves for each primer pair. Specificity was determent by checking the melting curve for a single peak. This indicates that each primer pair amplified a single amplicon (Fig. 2.10). The PCR product was then run on a 2% agarose gel to confirm the product size.

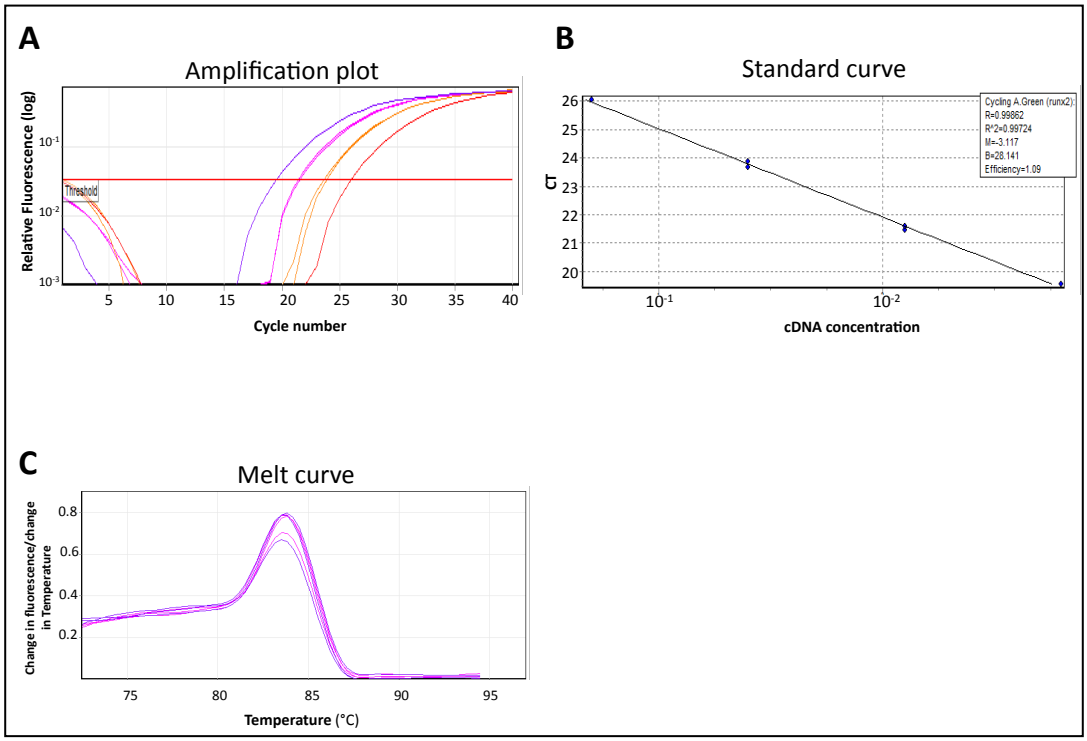


Figure 2.9**:** Quantitative PCR amplification data, constructed standard curve, and melting curve analysis for primer testing.

A) A representative image of a qRT-PCR run showing tight grouping of the triplicate amplification lines of each of the gene's standards. B) A standard curve analysis showing 109% efficiency, R^2=0.998, and slope=-3.11. C). A single peak in the melting curve analysis demonstrates that one specific product was amplified.

2.5.6.4 cDNA amplification and Semi quantitative RT- PCR

Diluted cDNA was used for the PCR reaction and SYBR green was used as a fluorescent dye to detect amplified double-stranded DNA specifications. Nuclease-free water (non template control) and no reverse transcription was used as negative controls, while E11.5-E14.5 and primary mouse calvaria osteoblast (POB) cDNAs were used as positive controls for cartilage and bone, respectively. The reaction mix was prepared as shown in Table 2.8, aliquoted in 0.2ml PCR tubes followed by 1ul of cDNA. PCR tubes were later run in a PCR machine (MJ Research, PTC200, peltier thermal cycler) using a three-step protocol with an annealing temperature at 55-60°C. The PCR cycles were repeated 29 times.

The generated amplicon was then run on a 1-1.5% agarose gel containing ethidium bromide to allow for visualisation of PCR product bands under UV light. A DNA ladder 100bp (Fermentas, SM0323) was run in same gel with tested samples to confirm PCR product size. The reaction mix was prepared as in Table 2.9, aliquoted in transparent q-PCR tubes followed by 1ul of cDNA. PCR tubes were run on a Rotor-Gene Q 6000 (Qiagen) machine using a two-step protocol as follows: 1-Denaturation: 95°C, 3 minutes. 2-Amplification: Denaturation 95°C, 5 seconds. Then combined anneling/extension 60°C, 1 second 3-Melting: Ramp from 65°C to 95°C rising by 1°C/step.). The PCR cycles were repeated 40 times. In most runs, the internal control (Gapdh) as well as negative controls (non-template and no reverse transcription) were included. Each PCR was performed in triplicates. The generated amplicon was then run on a 1.5-2% agarose gel as described above.

Reagent	**Stock Concentration**	**Volume/reaction (µl)**	**Company/ cat. no.**
Nuclease-free H_2O	-	32.75	Promega
Primer F	10um	0.5	See Table 2.10
Primer R	10uM	0.5	See Table 2.10
dNTPs	1mM	1	Promega/C114G
Buffer	5x	10	Promega/M531A
$MgCl_2$	25mM	3	Promega/A351B
GoTaq	5u/ul	0.25	Promega/M830B
cDNA	1:5 dilution	1	-
		Total volume =49	-

Table 2.8: Reaction mix for semi-quantitative RT-PCR (semi-qRT-PCR).

Reagent	Volume/reaction (ul)	Stock concentration	Company/ cat.no.
Forward primer	0.5	100uM (1:10 dilution)	See table 2.10
Reverse primer	0.5	100uM (1:10 dilution)	See table 2.10
Brilliant III Ultra-Fast SYBER@Green QPCR Master Mix	5	2x	Agilent/600882
cDNA	2-1	1:5 dilution	-
Nuclease-free H_2O	Top up to 10ul	-	Promega
Total volume	10		

Table 2.9: Reaction mix for quantitative RT-PCR (qRT-PCR).

2.5.6.5 PCR data analysis

Analysis of the PCR runs was performed using the Rotor-Gene Q 2.1.0.9 software. Quantification of gene expression was performed in the exponential range of each primer pair to get closer to 100% efficiency of primers and SYBR green. Then a comparative CT method was used to quantify changes in the expression of gene of interest between the different samples. Data plotted as ΔCT = CT (target gene) – CT (endogenous reference gene) whereby Gapdh was used as the reference gene. Primers used for gene expression analysis are listed in table 2.10.

2.5.7 Statistical analysis

All data were presented as the mean ± standard deviation (SD) from 3 replicates unless stated otherwise. The statistics used were the student's t-test (paired and unpaired) according to the comparison to test differences between to groups. For more than two groups a one-way ANOVA analysis was used with Holm-Sidak posthoc test for correcting multiple comparisons (GraphPad software, Prism 6) with a significant cut off point at $p \leq 0.05$. Data were represented as mean ± SD from a representative experiment. Experiments were repeated at least 3 times for verification unless stated otherwise. A cut off point at $p \leq 0.05$ was considered statistically significant.

Primer		Sequence 5'-3'	Accession number
Oct-4 (Pou5f1)	F	TTGTCCTCAGTGGGGCGGTT	NM_001252452.1
	R	CACCTTTCCAAAGAGAACGCCCAGG	
Nanog	F	AAGATGCGGACTGTGTTCTC	NM_028016.2
	R	CGCTTGCACTTCATCCTTTG	
Bry	F	CATGTACTCTTTCTTGCTGG	NM_009309.2
	R	GGTCTCGGGAAAGCAGTGGC	
Pdgfrα	F	CGCACGCCAGACTGTGTAT	NM_011058.2
	R	GTTAAAGACGGCACAGGTCAC	
Flk1 (KDR)	F	CACCTGGCACTCTCCACCTTC	NM_010612.2
	R	GATTTCATCCCACTACCGAAAG	
Sox17	F	AGAAACTGCAGACCAGAAGCTATCA	NM_011441.4
	R	GCT CATTGT ATC CAT GAG GTG ACA	
Gata1	F	CATTGGCCCCTTGTGAGGCCAGAGA	NM_008089.2
	R	ACCTGATGGAGCTTGAAATAGAGGC	
Nkx2.5	F	ATGCTGGCCGCCTTCAAGCC	NM_008700.2
	R	CTGCAGCGCGCACAGCTCTTT	
Tcf15 (Paraxis)	F	AAACCACTCCTGCGTTGTGTAAG	NM_009328.2
	R	TGGATGGCTAGATGGGTCCTT	
Tbx18	F	TAGACAGGAATCCATTTGCCAAA	NM_023814.4
	R	TAGTGATGGCCTCCAGAATGC	
Nkx3.2	F	AGATGTCAGCCAGCGTTTC	NM_007524.3
	R	AGGCGTAACGCTGTCATCCT	
Msgn1	F	TGGATTACAGCATGTTGGCTTT	NM_019544.1
	R	TCTCCGCTGGACAGACATCTT	
Tbx6	F	ATGTACCATCCACGAGAGTTGT	NM_011538.2
	R	CCAAATCAGGGTAGCGGTAAC	

Table 2.10: List of primer sequences used for PCR analysis.

(F: forward, R: reverse, PL: product length).

Primer		Forward	Accession number
Pax1	F	GCTCAGTACATTTGTTAATTTTGAAGAAC	NM_008780.2
	R	TTGCCCACCCCAATTCTTT	
Meox1	F	AGCGTCTTGTGTTCTCCAAGG	NM_010791.3
	R	ATGTGTGTGAACCTGGGAGGT	
Mesp1	F	TTTCCTTTGGTCTTGGCACCTTCG	NM_008588.2
	R	TCCAAGGAGGGTTGGAATGGTACA	
Foxf1a	F	CGGAGAAGCAGCCCTACT	NM_010426.2
	R	GCGCGCCTGAAGAAACTG	
Prrx1	F	AAAGAACTTCTCCGTCAGTCACC	NM_011127.2
	R	CCCACACTTTCGTCTGCTTGT	

Table 2.10: List of primer sequences used for PCR analysis (F: forward, R: reverse, PL: product length) (continued).

Primer		Forward	Accession number
Sox9	F	AGGTTTCAGATGCAGTGAGGAGCA	NM_011448.4
	R	CACAACACACGCACACATCCACAT	
Sox5	F	CCA TGG TGA CAA GCA GAC AG	NM_001243163.1
	R	AAC TTT ATT GCC ATC GAC TTC AT	
Col II (Col2a1)	F	CCAAACACTTTCCAACCGCAGTCA	NM_001113515.2
	R	AGTCTGCCCAGTTCAGGTCTCTTA	
Aggrecan	F	ATCCCACCCACATGGTGTCTTCTT	NM_007424.2
	R	TTAGATGCAGTTTGGGTGATGCGG	
ColX (Col10a1)	F	ACCCCAAGGACCTAAAGGAA	NM_009925.4
	R	CCCCAGGATACCCTGTTTTT	
GDF5	F	CCAAGAAGGATGAACCCAGA	NM_008109.2
	R	GGCAGATCCTGCTTTTGAAG	
Lubricin (Prg4)	F	GAA CCG CCG GCT GTG GAT GA	NM_021400.3
	R	TGT GGT GAC TTT GCT GTG TAGT	
Runx2	F	TGTTCTCTGATCGCCTCAGTG	NM_001145920.2
	R	CCTGGGATCTGTAATCTGACTCT	
Osterix (Sp7)	F	GGAGGCACAAAGAAGCCATACGC	NM_130458.3
	R	TGCAGGAGAGAGGAGTCCATTG	
ALP	F	TTGTGCGAGAGAAAGAGAGAGA	NM_007431.2
	R	GTTTCAGGGCATTTTTCAAGGT	
CoL I (col 1 a1)	F	CTTGGTGGTTTTGTATTCGATGAC	NM_007742.3
	R	GCGAAGGCAACAGTCGCT	
BSP	F	GCTACTTTCTTTATAAGCATGCCTACT	NM_008318.3
	R	GCCTCCCTGGACTGGAAAC	
OC (Bglap2)	F	CTCACAGATGCCAAGCCCA	NM_001032298.2
	R	CCAAGGTAGCGCCGGAGTCT	
Gapdh	F	CCATGGAGAAGGCTGGGG	NM_002046.5
	R	CAAAGTTGTCATGGATGACC	

Table 2.10: List of primer sequences used for PCR analysis (F: forward, R: reverse, PL: product length) (continued).

Chapter 3 Effect of ROCK inhibition on mesoderm enrichment and specification of chondrocyte and osteoblast lineages

3.1 Introduction

During embryonic development, mesodermal chondrogenic and osteogenic precursor cells (or skeletogenic cells) arise from a common mesenchymal-type stem cells (MSCs) (Olsen et al. 2000; Lefebvre & Bhattaram 2010), which emerge from paraxial and lateral plate mesoderm depending on the anatomical location of cartilage and bone tissue. Mesoderm is derived from the primitive streak during the gastrulation. The differentiation process of each cartilage and bone lineage is controlled by lineage-specific transcription factors and signalling pathways that modify their expression as been mentioned in the Introduction section 1.5 and 1.6.

The RhoA/ROCK signalling pathway proved to have a role in controlling skeletal lineage decisions (Sordella et al. 2003; McBeath et al. 2004) and later differentiation stages (Harmey et al. 2004; Woods et al. 2005; Prowse et al. 2013). However, reports showed contradictory results owing to the complexity of the pathway and other factors related to culture conditions such as the culture model used, cell origin and developmental stage of the cells (Harmey et al. 2004; McBeath et al. 2004; Woods & Beier 2006). Thus, the molecular mechanism of Rho/ROCK signalling in influencing chondrocyte and/or osteoblast commitment is not yet clear. Therefore, in this chapter, the 3-phase differentiation scheme of ESC differentiation described here gives an opportunity to investigate the role of this signalling pathway in a chemically defined, serum free, step-wise method.

The ESC/EB culture model used in this project relies on the formation of a primitive streak-like population, and the induction of a mesoderm population. Gadue et al. (2006) established, using ESC *in vitro* culture, that the activation of Activin/Nodal and Wnt signalling is required for primitive streak/mesoderm (Bry positive) induction. Later on, Gouon-Evans et al. (2006) showed that ESC cultures were enriched for mesodermal subtypes by the activation of FGF signalling by FGF2 treatment, which had previously been established in Xenopus (Fletcher & Harland 2008). Recently, Craft et al. (2013) showed that

using a 3-phase ESC/EB protocol including mesoderm induction by activation of Activin A/Nodal and canonical Wnt and simultaneous inhibition of BMP signalling by Noggin, efficiently enriched for a mesoderm-derived population with chondrogenic potential.

Hence, the objectives of this chapter are: firstly, to establish and optimise the early steps of ESC differentiation of mesoderm towards both chondrocyte and osteoblast differentiation; secondly, to investigate the molecular profile during mesoderm induction (phase I) and mesoderm enrichment (phase II) phases; and thirdly, to investigate the role of the inhibition of ROCK on mesoderm enrichment and on both cartilage and bone lineage specification.

3.2 Methods

For ESCs differentiation towards mesoderm, a serum free, 3-phase ES/EB differentiation culture model was employed, using specific recombinant growth factors (Activin A, Wnt3a and Noggin) as indicated in chapter 2 section 2.1.3.

Briefly, in the absence of LIF, ESCs form EBs in suspension cultures. In the first phase (phase I), EBs were cultured in mesoderm induction media containing (9 ng/ml) Activin A, Wnt3a (25ng/ml), and Noggin (100 ng/ml) for 1 or 2 days. In the second phase (phase II), EBs were cultured in mesoderm enrichment media containing FGF2 (10ng/ml) for 3 days. The role of ROCK inhibition was analysed in phase II by using the ROCK inhibitor Y27632 (10uM). Analysis was undertaken by monitoring morphological changes and molecular analysis of genes representing mesodermal subpopulations (paraxial and lateral plate mesoderm), cartilage and bone lineages, using RT-PCR and qPCR. This was followed by functional differentiation analysis of chondrocyte and osteoblast differentiation, which was assessed by histochemical staining, Alcian blue (AB) to indicate chondrocyte and Alkaline Phosphatase (ALP) to indicate osteoblast differentiation.

3.3 Results

3.3.1 Morphological analysis of directed ESC differentiation via generation of Embryoid bodies (EBs)

During the 3-phase differentiation process, cells undergo specific morphological changes. Prior to differentiation, the undifferentiated ESCs (CCE cell line) grew in feeder-independent conditions as colonies (Fig. 3.1B), showing a typical ESC morphology with a large nucleus to cytoplasm ratio (Fig. 3.1C). ESCs committed for differentiation formed EBs in a suspension culture in the presence of induction medium (phase I) (Fig. 3.1D,E). After that EBs were dissociated and re-aggregated in suspension cultures to reform EBs again (phase II) (Fig. 3.1F-I). In phase III, cells exhibited a typically fibroblastic appearance when plated as single cells in a monolayer culture (Fig. 3.1 J,K).

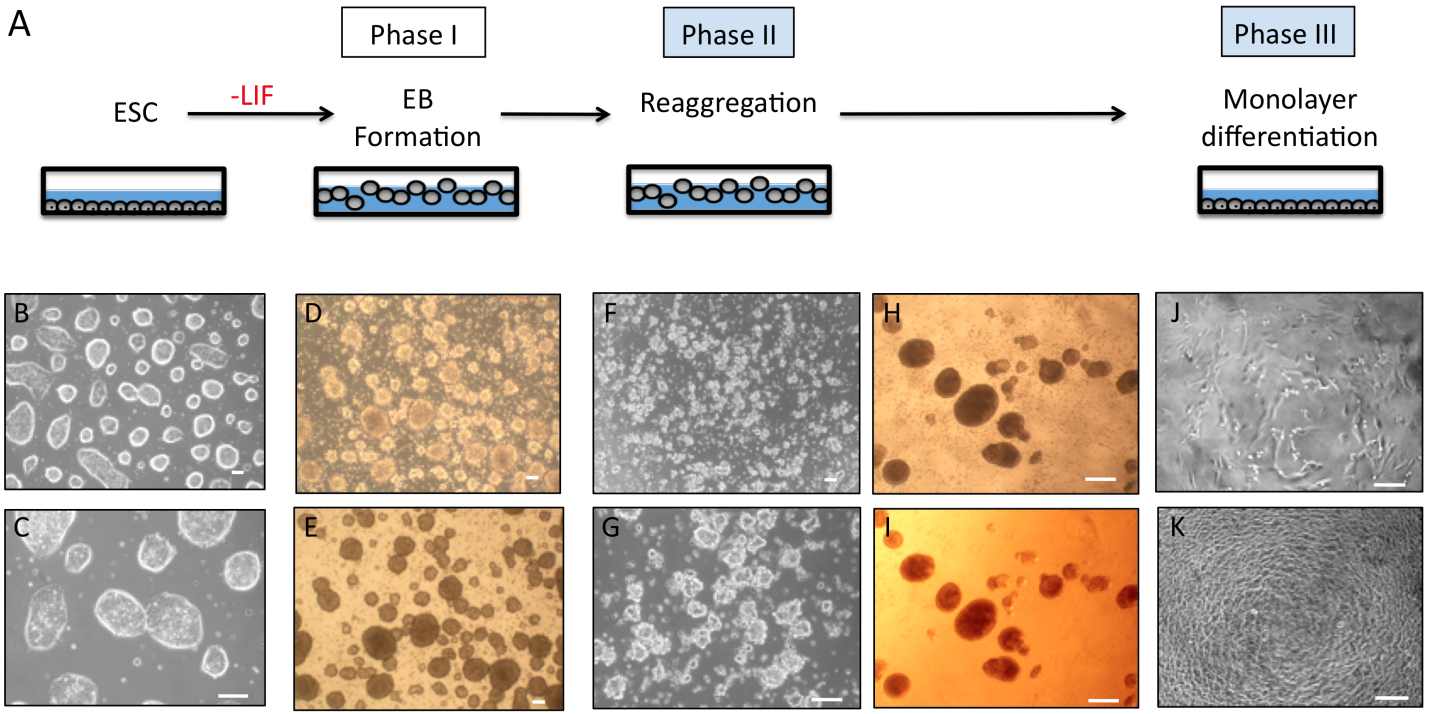


Figure 3.1: The 3-phase, serum-free, culture model for directed differentiation of ESCs through the generation of embryoid bodies (EBs). A) ESCs are committed to differentiation when cultured in the absence of LIF. The formed EBs were replated as singlecells and cultured in mesoderm induction media (Activin A, Wnt3a, Noggin) (phase I). After that, EBs were dissociated and plated as single cells and cultured in mesoderm enrichment media (phase II), followed by replating as single cells in a monolayer culture for chondrogenic or osteogenic differentiation (phase III). B&C) Undifferentiated ESC colonies. Phase-contrast (D) and bright field (E) images of Embryoid bodies (EBs) in phase I. F&G) Phase-contrast micrographs of reaggregates in phase II 24h after plating. H) Bright-field micrographs of reaggregates in phase II 48h after plating. (I) Phase contrast of reaggregates in phase II 48h after platting. J&K) Fibroblastic differentiation in a monolayer culture in phase III. Phase-contrast images of subconfluent (J) and confluent (K) monolayer cultures in phase III. All images are from representative experiments (n=21). Scale bar: 100 µm. Detailed for each phase present in figure 2.1 in chapter 2.

3.3.2 Optimisation of the mesoderm induction period (phase I)

In this project, the 3-phase ES/EB culture model was first optimised to investigate the differentiation of the specific CCE ESC line towards both the cartilage and bone lineages simultaneously. For this reason, the differentiation of ESCs towards both cartilage and bone lineages was examined using the same mesoderm induction cocktail and 1-day mesoderm induction period as used by Craft et al. (2013), compared to a longer, 2-day induction period (Fig, 3.2 A,B).

The Alcian blue and ALP staining results from the 1-day mesoderm induction period (2-3d) showed negative Alcian blue staining (Fig. 3.2C) but positive ALP staining (Fig. 3.2E). These results suggest that the CCE ESCs differentiated primarily toward the osteogenic rather than the chondrogenic lineage under these conditions.

On the other hand, the results from the 2-day mesoderm induction period (2d-4d) showed positive staining for both Alcian blue (Fig. 3.2D) and ALP (Fig. 3.2F), representing differentiation towards both the chondrogenic and osteogenic lineages simultaneously. As the aim of this project was to study the effect of ROCK inhibition on both cartilage and bone lineage development, all experiments for the remainder of project were performed using a 2-day mesoderm induction protocol.

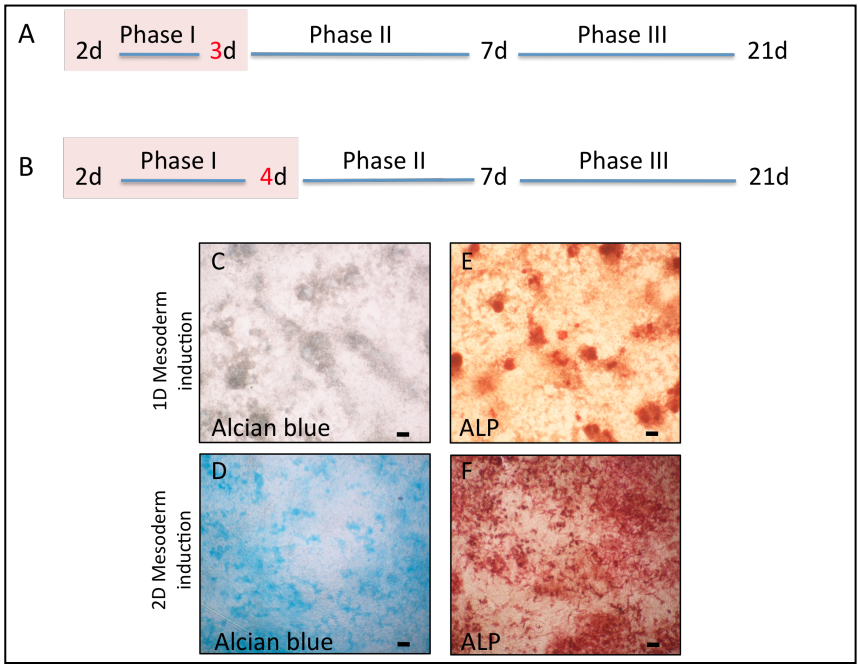


Figure 3.2: The effect of a 1-day verses 2-day mesoderm induction phase (phase I) on the chondrogenic and osteogenic differentiation of ESCs.

ECSs were cultured in mesoderm induction media for 1 day (A) or 2 days (B), then re-plated for mesoderm enrichment for 3 days followed by re-plating in a monolayer culture for differentiation. Cultures were fixed after 2 weeks for staining. Representative image of Alcian blue stained monolayers: (C) from a 1-day (1D) and (D) from a 2-day (2D) mesoderm induction period. Representative image of ALP stained monolayers: (E) from a 1-day and (F) from a 2-day mesoderm induction period. The data are representative from 3 independent experiments. Scale bar: 100 μm.

3.3.3 Molecular analysis of 2-day mesoderm induction phase (phase I)

Monitoring mesoderm induction entailed analysing the expression of Brachury (Bry), which is a definitive marker for primitive streak and early mesoderm. A time course analysis (Fig. 3.3A) using RT-PCR showed that Bry expression was first detected between 2-4d of induction and this was maintained up to 6d (Fig. 3.3B). Further characterisation in detail using qPCR showed a ~ 8-fold increase in Bry expression compared to control 2d cultures (Fig.3.3C). This suggests that under the used induction conditions ESCs are committed to express the essential primitive streak/early mesoderm marker, which is Bry gene.

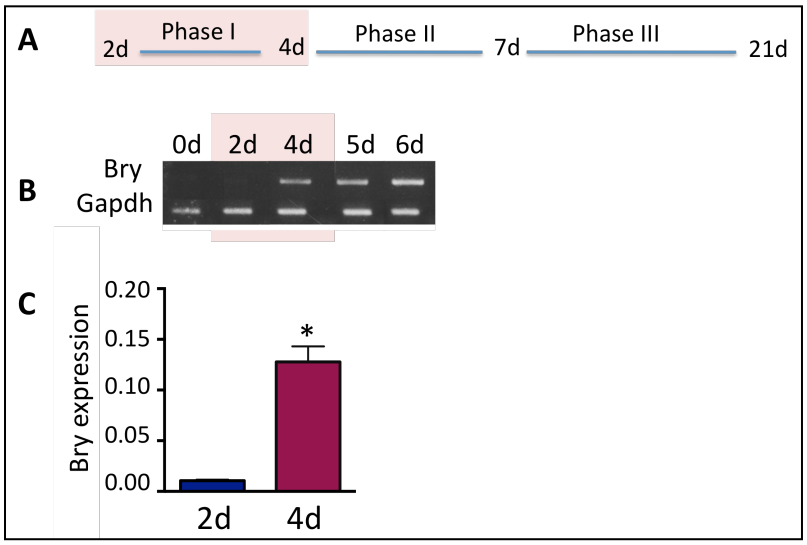


Figure 3.3: The expression of Brachyury (Bry) at the primitive streak/mesoderm induction phase (phase I).
A) A scheme showing the time points of each differentiation phase. RNA was extracted from 0d (undifferentiated ESCs), 2d (prior to mesoderm induction), 4d (after mesoderm induction), 5d and 6d mesoderm-induced EBs. B) RT-PCR analysis of Bry expression after 2-day (2-4d), 3-day (2-5d) and 4-day (2-6d) induction periods. C) Quantitative PCR analysis of Bry expression after a 2-day mesoderm induction. Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (paired student t-test; * $p < 0.05$, 4d vs 2d).

To further confirm ESC differentiation towards mesoderm, qPCR analysis showed a 2-fold down regulation in the expression of Oct-4 (Pou5f1) (pluripotency marker) and Sox17 (endoderm marker) at the mesoderm induction phase (phase I) (Fig. 3.4). These results suggest that ESCs differentiated towards the mesodermal lineage that was distinct from the endoderm lineages present in anterior primitive streak.

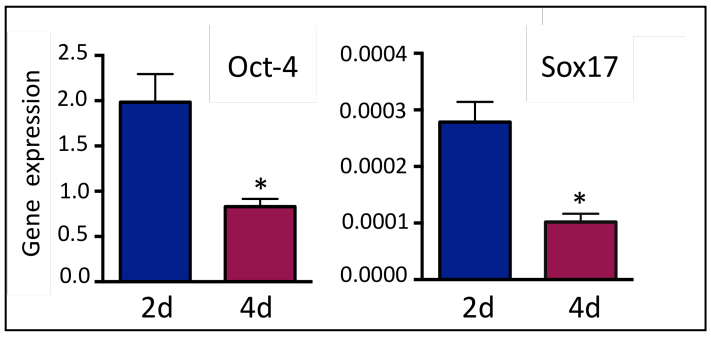


Figure 3.4: The expression of Oct4 (pluripotency marker) and Sox17 (endoderm marker) at the mesoderm induction phase (phase I).
Quantitative PCR analysis of Oct-4 and Sox17 expression at the mesoderm induction phase (phase I). Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (paired student's t-test; * $p < 0.05$, 4d vs 2d).

3.3.4 Molecular analysis of mesodermal subpopulation (paraxial and lateral plate mesoderm) marker gene expression

Following the differentiation of ESCs to a mesoderm population expressing Bry, first it was investigated whether a further 3d FGF2 treatment phase (Fig. 3.5A) would enrich for the differentiation of Bry positive mesoderm population, as FGF2 been known to maintain mesoderm expression (Fletcher & Harland 2008). Quantitative PCR analysis of Bry expression showed a 2.5-fold increase in Bry expression in 7d cultures compared to 4d cultures (Fig. 3.5B). This was accompanied by a 3.7-fold reduction in Oct-4 expression in 7d cultures in comparison to 4d cultures (Fig. 3.5B). Therefore, the additional 3d treatment with FGF2 during mesoderm enrichment phase further increased the proportion of primitive streak/mesoderm populations with a further decrease in pluripotency markers.

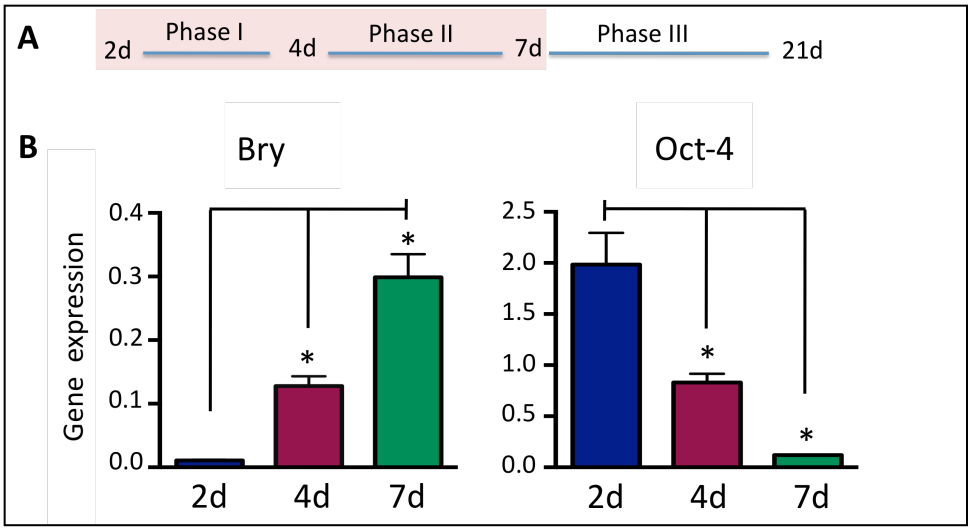


Figure 3.5: The effect of the mesoderm enrichment phase (phase II) on Bry and Oct-4 expression.

Quantitative PCR analysis of Bry and Oct4 gene expression during mesoderm induction (phase I) and mesoderm enrichment (phase II) phases of differentiation. Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (paired student's t-test; * $p < 0.05$, 2d vs 7d and 4d vs 7d).

It was next investigated whether the 3d FGF2 treatment in the mesoderm enrichment phase (phase II) led to paraxial mesoderm formation and differentiated towards somitic mesoderm, from which cartilage and bone develop. Therefore, the expression of (Pdgfrα, Tbx6 and Msgn1) that are expressed during paraxial mesoderm and somite development were analysed by qPCR. Culture of cells in FGF2 for 3 days (4-7d) in phase II caused a 7.5-fold increase in Pdgfrα expression, as well as a 6-fold and a ~2-fold increase in the expression of Tbx6 and Msgn1 expression, respectively compared to 2d and 4d cultures (Fig. 3.6). These results suggest that FGF2 treatment enriched for paraxial/pre-somitic mesoderm differentiation at the mesoderm enrichment phase (phase II).

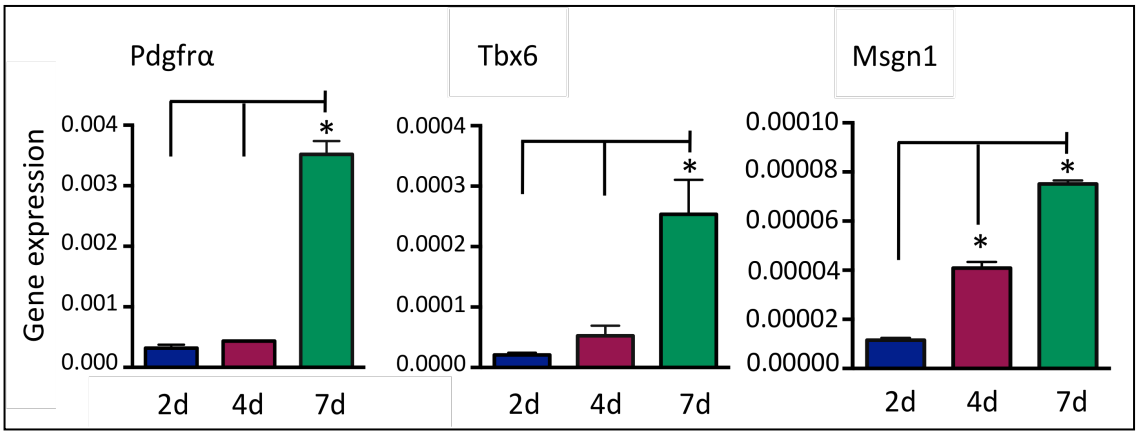


Figure 3.6: The expression of paraxial mesoderm markers at the mesoderm enrichment phase (phase II).

Quantitative PCR analysis of Pdgfrα, Tbx6 and Msgn1 (paraxial mesoderm/pre-somitic markers) expression at the mesoderm induction (phase I) and mesoderm enrichment (phase II) phases of differentiation. Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (paired student's t-test; * $p < 0.05$, 2d vs 7d and 4d vs 7d).

Further investigation was then conducted on the expression of additional paraxial/pre-somitic/somitic mesoderm markers, such as Tcf15, Tbx18, Nkx3.2, Tbx6, Pax1, Msgn1 and Meox1. The results showed a significant increase in the expression of all markers at 7d compared to 4d cultures (Fig. 3.7B). These results further confirm that FGF2 treatment enriched for paraxial mesoderm differentiation.

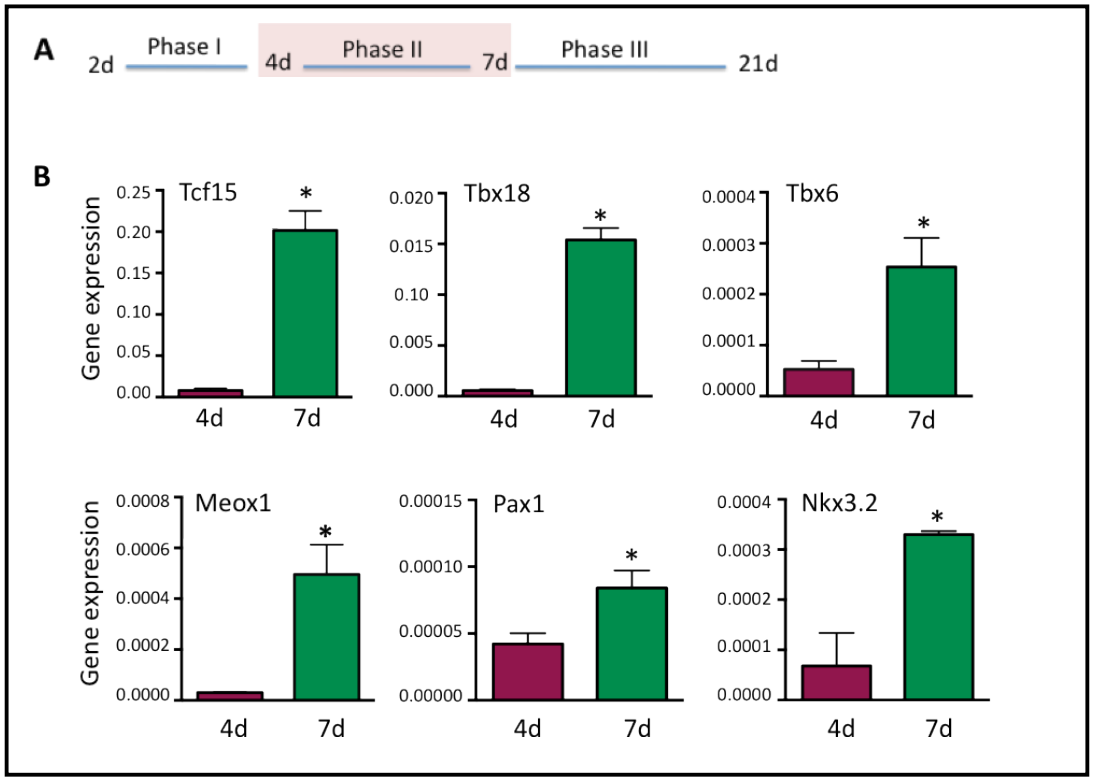


Figure 3.7: The expression of paraxial mesoderm, pre-somitic and somitic markers at the mesoderm enrichment phase (phase II).

Quantitative PCR analysis of the paraxial mesoderm/pre-somitic/somitic markers Tcf15, Tbx18, Nkx3.2, Tbx6, Pax1, Msgn and Meox1 at the mesoderm induction phase (phase II). Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (paired student's t-test; * $p < 0.05$, 4d vs 7d).

During development, cartilage and bone originate not only from paraxial mesoderm but also from lateral plate mesoderm, which is the origin of limb bud mesenchyme (chapter 1, section 1.3.3). Therefore, it was subsequently investigated whether the 3-day FGF2 treatment in the mesoderm enrichment phase (phase II) differentiated the cells to lateral plate mesoderm lineage. Quantitative PCR results showed an upregulation in the expression of Flk-1, Foxf1a, Mesp1 and Prrx1 at 7d compared to 4d cultures (Fig. 3.8), suggesting that FGF2 treatment stimulated the differentiation of lateral plate mesoderm at the mesoderm enrichment phase (phase II).

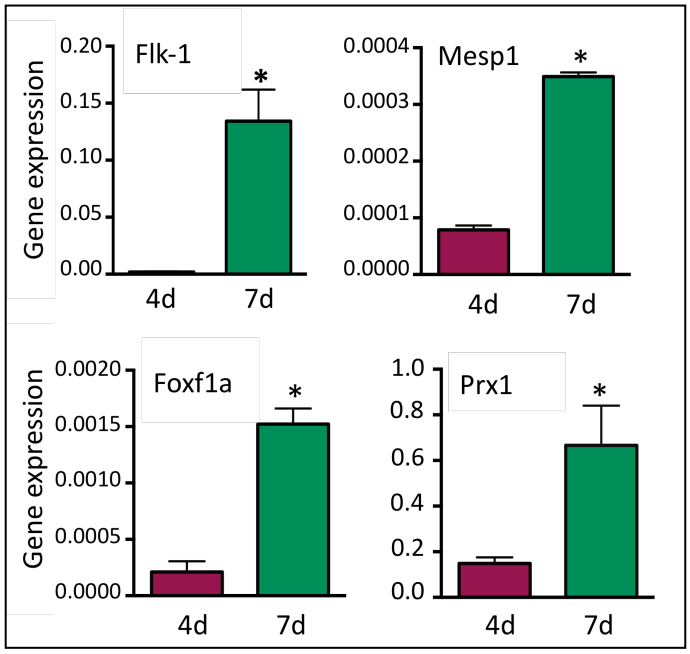


Figure 3.8: The expression of lateral plate mesoderm markers at the mesoderm enrichment phase (phase II).

Quantitative PCR analysis of lateral plate mesoderm marker expression (Flk-1, Foxf1a, Mesp1, Prrx1) at the mesoderm induction phase (phase II). Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (paired student's t-test; * $p < 0.05$, 4d vs 7d).

It was then necessary to confirm the specificity of mesoderm enrichment by investigating the expression of more posterior primitive streak markers. Semi-quantitative PCR analysis showed the lack of haematopoietic mesoderm (Gata1) and cardiac mesoderm (Nkx2.5) marker expression (Pevny et al. 1991; Komuro & Izumo 1993) during differentiation whilst also confirming again the decrease in expression of the pluripotency marker, Nanog, and activation of Bry and Flk-1 expression (Fig. 3.9). These results suggest that the mesoderm induction and enrichment conditions were sufficient for expression of the paraxial and lateral plate mesoderm subpopulations from where cartilage and bone are thought to arise.

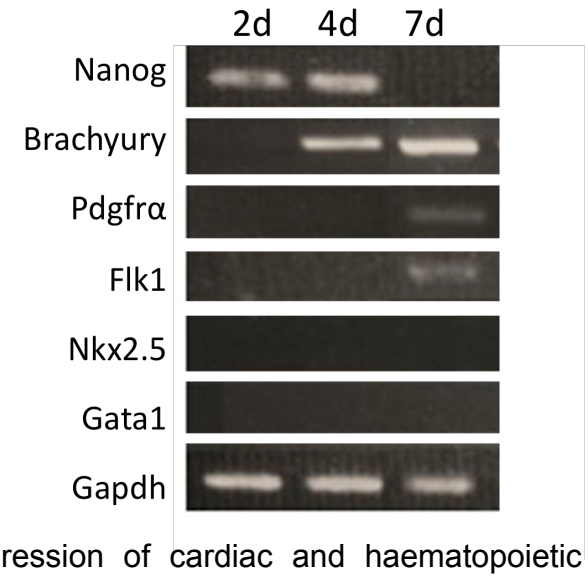


Figure 3.9: The expression of cardiac and haematopoietic markers at the mesoderm induction (phase I) and mesoderm enrichment phases (phase II).

A semi-quantitative PCR analysis of Nanog, Bry, Pdgfrα, Flk-1, Nkx2.5 (cardiac marker) and Gata1 (haematopoietic marker) expression at the mesoderm induction (phase I) and mesoderm enrichment phase (phase II). Data are from a representative experiment from 3 independent experiments.

3.3.5 Chondrogenic and osteogenic lineage specification at the mesoderm enrichment phase (phase II)

During embryonic development, cartilage and bone differentiation are controlled by the expression of key transcription factors (Lefebvre et al. 1997; Smits et al. 2001; Otto et al. 1997; Nakashima et al. 2002). To investigate the changes in expression of chondrogenic and osteogenic transcription factors during the mesoderm enrichment phase, the expression of Sox9 and Sox5 was analysed for cartilage differentiation, and Runx2 and Osx for bone differentiation. The results showed a significant increase in the expression of both cartilage and bone markers compared to 4d cultures (Fig. 3.10). Therefore, these results suggest that the cells at the end of the mesoderm enrichment phase (phase II) have both chondrogenic and osteogenic potentials, i.e. containing cells representing a bi-potential chondro-osteoprogenitor population, or separate populations. The potential of these cells to differentiate further into chondrocytes and osteoblasts, respectively, will be investigated *in vitro* and *in vivo* next chapters 4, 6, and 7, respectively.

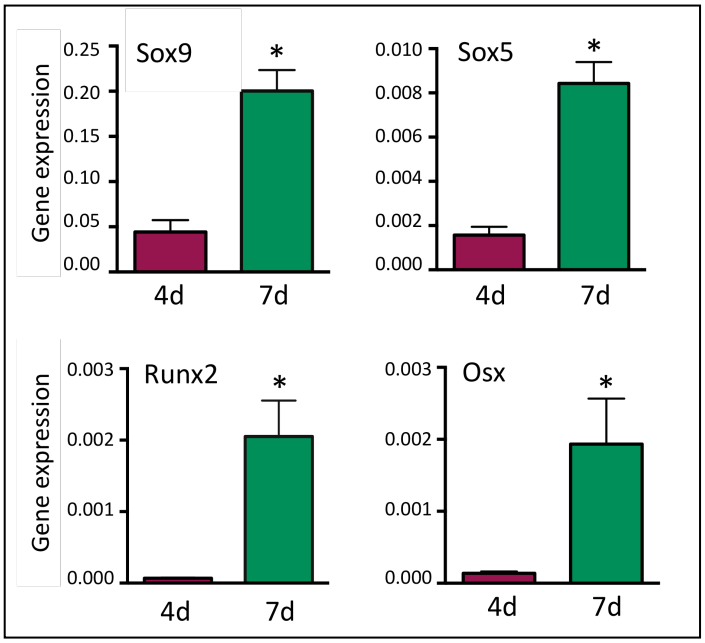


Figure 3.10: The expression of both chondrogenic and osteogenic lineage transcription factors at the mesoderm induction phase (phase II).

Quantitative PCR analysis of (A) Chondrogenic marker (Sox9 and Sox5) and (B) Osteogenic marker (Runx2 and Osx) expression at the mesoderm enrichment phase (phase II). Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (paired student's t-test; * $p < 0.05$, 4d vs 7d).

3.3.6 Effect of ROCK inhibition on paraxial and lateral plate markers expression during the mesoderm enrichment phase (phase II).

To explore the effect of ROCK inhibition on ESC-derived mesoderm commitment towards the chondrogenic and/or osteogenic lineages, first the effect of ROCK inhibition on paraxial and lateral plate mesoderm marker gene expression was investigated. To this end, cells were cultured in FGF2 for 3 days (4d-7d) in the presence and absence of the ROCK inhibitor Y27632 (Fig.3.11A). The results showed that Y27632-treatment led to a significant increase in the expression of Pdgfrα, Tcf15, Tbx18, Pax1, Msgn1 and Tbx6 compared to 4d cultures (Fig.3.11B), while Pax1 expression was reduced upon Y27632 treatment at 7d (7d+Y) compared to 4d cultures (Fig.3.11B). In addition, in comparison to Y27632-untreated cultures (7d-Y), Y27632 treatment (7d+Y) demonstrated a downregulation in the expression of all genes except Meox1, whereby Y27632 treatment showed no effect on its expression compared to Y27632-untreated cultures (7d-Y) (Fig. 3.11B). These results suggest that

inhibition of ROCK signalling directed the differentiation of ESC-derived mesoderm towards paraxial mesoderm population at reduced levels. Thus, it seems that ROCK signalling controls paraxial mesoderm in a distinctive manner.

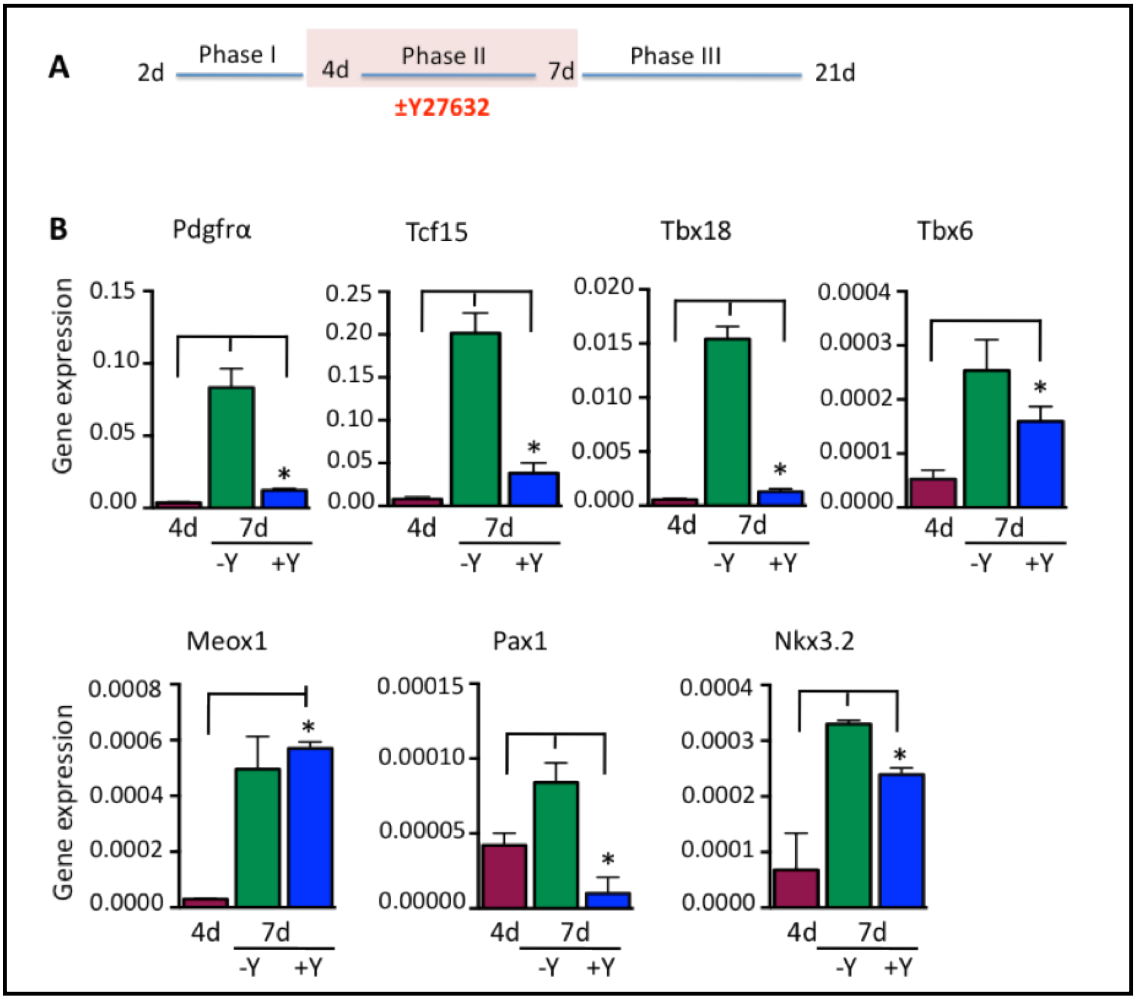


Figure 3.11: Effect of ROCK inhibition on the expression of paraxial mesoderm markers at the mesoderm enrichment phase (phase II).

Quantitative PCR analysis of the paraxial mesoderm marker genes, Pdgfrα, Tcf15, Tbx18, Nkx3.2, Tbx6, Pax1, Msgn1 and Meox1, at the mesoderm enrichment phase (phase II) (4d-7d) in the presence and absence of Y27632 (±Y). Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (* $p < 0.05$, (unpaired student's t-test; 7d-Y vs 7d+Y) and (paired student's t-test; 4d vs 7d+Y).

Furthermore, analysis of Flk-1, Foxf1a, Mesp1 and Prx1 expression was conducted to find out whether ROCK inhibition also affects the expression of lateral plate mesoderm markers. The results showed that Y27632 treatment upregulated the expression of both Flk-1 and Prx1 at 7d compared to 4d cultures, while Y27632 treatment had no effect on the expression of either Mesp1 or Foxf1a at the same time point (Fig. 3.12). In addition, in comparison to Y27632-untreated cultures (7d-Y), Y27632 treatment demonstrated that Y27632 treatment (7d+Y) downregulated the expression of all tested lateral plate mesoderm markers (Flk-1, Foxf1a and Mesp1) compared to untreated cultures (7d-Y) (Fig. 3.12). These results suggest that inhibition of the ROCK pathway may be differentiating the ESC-derived mesoderm towards a distinctive mesoderm population with reduced lateral plate mesoderm marker expression.

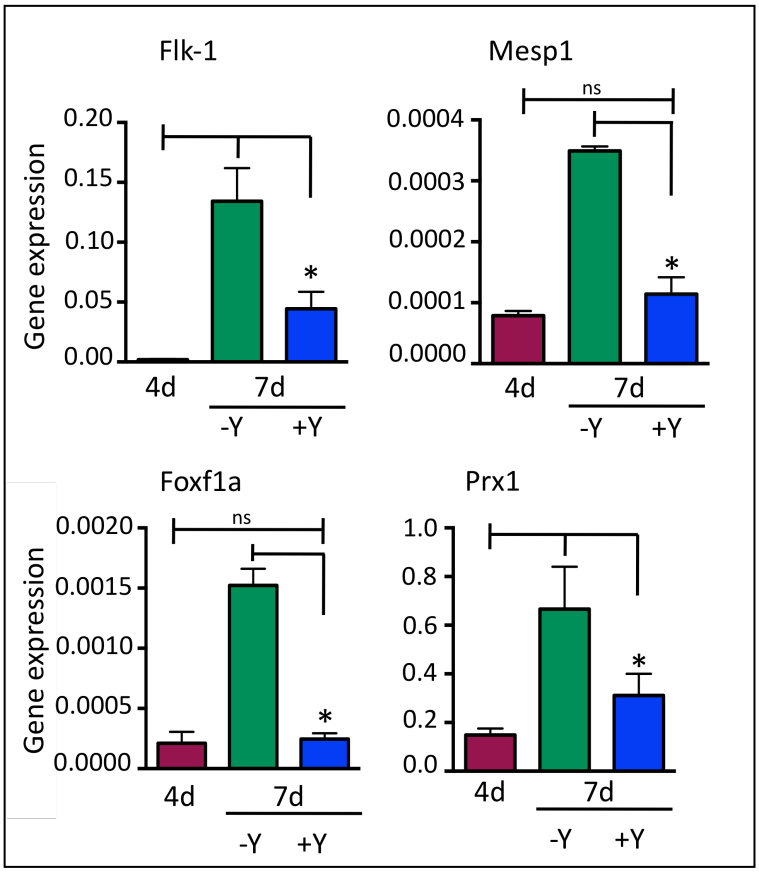


Figure 3.12: Effect of ROCK inhibition on the expression of lateral plate mesoderm markers at the mesoderm enrichment phase (phase II).

Quantitative PCR analysis of the lateral plate mesoderm marker genes, Flk-1, Foxf1a, Mesp1 and Prx1 at the mesoderm enrichment (phase II). Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (* $p < 0.05$ (unpaired student's t-test; 7d-Y vs 7d +Y) and (paired student's t-test; 4d vs 7d+Y).

3.3.7 Effect of ROCK inhibition on the expression of cartilage and bone transcription factors at the mesoderm enrichment phase (phase II)

To further investigate the effect of ROCK inhibition on ESC-derived mesoderm commitment towards the chondrogenic and osteogenic lineages, cartilage and bone lineage transcription factors were also assessed following addition of Y27632 to the mesoderm enrichment phase for 3 days (4-7d). The result revealed that Y27632 treatment (7d+Y) upregulated both cartilage and bone markers when compared to 4d cultures (Fig. 3.13A). In contrast, when compared to Y27632-untreated cultures (7d-Y), results demonstrated that Y27632 treatment downregulated Sox9 and Osx expression while upregulating Sox5 expression by more than 2-fold (Fig. 3.13A). Sox9 expression was also verified at the protein level, revealing a strong immunofluorescence staining in Y27632-untreated cells compared to treated cells (Fig. 3.13B), suggesting that ROCK inhibition attenuated chondrogenic and osteogenic lineage commitment at the mesoderm enrichment phase (phase II). Taken together the results suggest that inhibition of ROCK signalling enriches for both chondrogenic and osteogenic lineages at the mesoderm enrichment phase, however, this enrichment is at a lower level than under basal conditions.

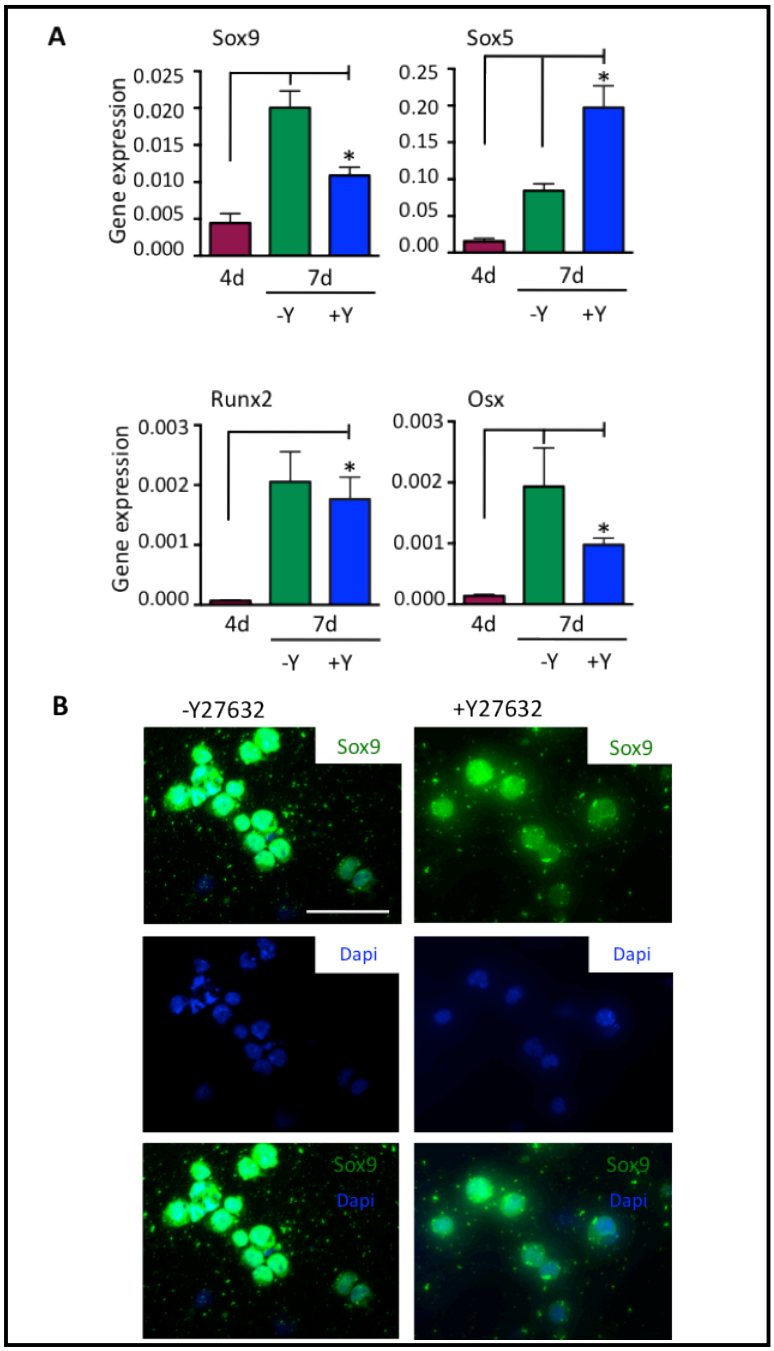


Figure 3.13: The effect of ROCK inhibition on the expression of chondrogenic and osteogenic transcription factors at the mesoderm enrichment phase (phase II).

Quantitative PCR analysis of (A) chondrogenic markers (Sox9 and Sox5) and (B) osteogenic markers (Runx2 and Osx). Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (* $p < 0.05$, (unpaired student's t-test; 7d-Y vs 7d+Y) and (paired student's t-test; 4d vs 7d +Y). (B) Immunofluorescence staining following cytospin, showing Sox9 expression in Y27632-treated and untreated cultures at the mesoderm enrichment phase (phase II). Images are from a representative image from one experiment. Scale bar: 100um.

3.4 Discussion

In this chapter, the objective was to investigate the role of inhibiting ROCK signalling during the mesoderm enrichment phase (phase II) on both sum-mesoderm and cartilage/bone specification. This was achieved in four steps. The first step was optimisation of the kinetics of primitive streak induction in the specific ESC line used in this study, followed by differentiation towards the cartilage and bone lineages. Secondly, the expression of primitive streak and mesodermal markers during the mesoderm induction (phase I) and enrichment (phase II) phases was investigated. Thirdly, at phase II, the cartilage and bone lineages specification potential was investigated. Fourthly, the effect of inhibiting the ROCK pathway on both mesodermal subpopulations and cartilage and bone-specific lineage markers was investigated.

The findings suggest that the mesoderm enrichment phase enriched for the expression of both paraxial and lateral plate sub-mesodermal populations, expressing both cartilage and bone transcription factors. In addition, the inhibition of ROCK signalling at phase II directed ESC-derived mesoderm differentiation towards a distinctive paraxial and lateral plate sub-mesoderm populations. Moreover, ROCK signalling inhibition directed ESC-derived mesoderm differentiation towards a distinctive chondrogenic and osteogenic populations by differentially regulating the expression of paraxial/lateral plate mesoderm and cartilage/ bone transcription factors, suggesting that ROCK signalling might be a key modulator in the commitment of mesodermal-derived chondro-osteoprogenitor progenitor populations. These findings will be further discussed in the following sections.

3.4.1 Mesoderm induction (Phase I: 2-4d)

A 3-phase ES/EB culture model has been published by our collaborator recently for mesoderm-derived chondrogenic differentiation (Craft et al. 2013). In this project, this differentiation protocol was optimised for the differentiation of the CCE cell line towards both the chondrogenic and osteogenic lineages simultaneously. This was achieved by using the same induction cocktail of growth factors, but extending the mesoderm induction to a 2-day instead of a 1-day period, and this was evident by the histochemical staining for cartilage and

bone. Mesoderm induction was confirmed by Bry expression, which is a primitive streak and early mesoderm marker (Wilkinson 1990; Yamaguchi et al. 1999; Kahle et al. 2010).

It is well-established that the concentration of Activin can direct primitive streak development towards mesodermal and endodermal lineages representative of posterior and anterior primitive streak (Green et al. 1992; Kubo et al. 2004). The induction protocol used here was sufficient for generating a mesodermal population that is predicted to give rise to mesenchymal cell types in cartilage and bone (discussed later on). This was demonstrated in several ways. First, the pluripotency gene, Oct-4 (Nichols et al. 1998) was downregulated after mesoderm induction (at 4d) and then further after mesoderm enrichment (at 7d) phases of differentiation. Second, under the same conditions, Sox17 an endoderm marker (Kanai-Azuma et al. 2002) was downregulated at 4d. Third, haematopoietic and cardiac markers were also not expressed at 4d and 7d, as shown by GATA1 and Nkx2.5 markers (Pevny et al. 1991; Komuro & Izumo 1993; Chen & Schwartz 1995). The lack of GATA1 and Nkx2.5 gene expression was likely due to inhibition of BMP signalling with Noggin, since it is known that BMP signalling is important for haematopoiesis differentiation (Johansson & Wiles 1995; Sumi et al. 2008; Nostro et al. 2011). Indeed, a preliminary experiment confirmed haematopoietic (osteoclasts) and cardiac lineages (beating cardiomyocytes) in CCE cells when BMP was used in the induction phase (phase I: 2-4d) rather than Noggin (personal communication). Taken together, the induction conditions appear to be sufficient for further enrichment and differentiation of cartilage and bone lineages.

3.4.2 Mesoderm enrichment (Phase II: 4-7d)

Cartilage and bone largely develop from two mesodermal subpopulations, paraxial mesoderm and lateral plate mesoderm. Axial bone develops from paraxial mesoderm while appendicular bone develops from lateral plate mesoderm (Olsen et al. 2000; Zhang et al. 2009; Keller & Nieden 2011). In this project, Bry-expressing differentiated cells expressed paraxial mesoderm by the expression of Pdgfrα at the mesoderm enrichment phase (phase II). Pdgfrα is expressed in paraxial mesoderm and somites (Orr-Urtreger et al. 1992; Takakura et al. 1997). In addition, cells in phase II expressed pre-somitic markers such as Tbx6 and Msgn1, which are also downstream markers of Wnt

signalling (Chalamalasetty et al. 2011). The paraxial mesoderm population in phase II was further marked by expression of genes required for temporal development of somites/sclerotome, such as Tcf15, Tbx18, Meox1, Pax1 and Nkx3.2. During development, paraxial mesoderm segments into somites (Tam & Tan 1992; Kulesa & Fraser 2002b), which are marked by upregulation of Tcf15 and Tbx18 somitic mesoderm markers (Burgess et al. 1996; Bussen et al. 2004; Singh et al. 2005). Furthermore, specification of the sclerotome is marked by the expression of Meox1 (Mankoo et al. 2003; Rodrigo et al. 2004), Pax1 (Dietrich et al. 1993; Rodrigo 2003) and Nkx3.2 (also known as Bapx1) (Tribioli et al. 1997; Tribioli & Lufkin 1999).

In addition, during phase II, the mesodermal population expressed Flk-1 together with Pdgfrα. Flk-1 is expressed in lateral plate mesoderm that gives rise to somatopleuric mesoderm, the origin of limb bud chondrogenic mesenchymal cells (Lawson et al. 1991; Yamaguchi et al. 1993; Kabrun et al. 1997; Kinder et al. 1999) as it is expressed in the haematopietic mesoderm (Takakura et al. 1997). The lateral plate mesoderm population in phase II was further marked by the expression of other lateral plate mesoderm markers such as Mesp1 (Saga et al. 1996; Chan et al. 2013) and Foxf1a (Chang & Ho 2001; Ormestad et al. 2004) as well as Prx1, which is expressed during limb bud development (Martin et al. 1995; Martin & Olson 2000).

At the mesoderm enrichment phase (phase II), both Sox9 and Sox5 gene expression were upregulated (Lefebvre et al. 2001), suggesting commitment towards the chondrogenic lineage. In addition, the immunofluorescence staining confirmed Sox9 expression at the protein level. Sox9 is a key transcription factor marking chondrogenic commitment and differentiation (Bi et al. 1999). Furthermore, Sox9 expression is not only a chondrogenic marker, but also a chondro-osteoprogenitor population marker (Akiyama et al. 2002; Akiyama et al. 2005). Therefore, expression of Sox9 at the mesoderm enrichment phase (phase II) could also be suggestive of the presence of an osteogenic population. Thus, osteoblast transcription factors were also investigated in phase II.

The expression of both Runx2 and Osterix were upregulated at phase II. Runx2 is a key transcription factor (Komori et al. 1997; Otto et al. 1997) and Osterix is

a downstream marker for osteogenic commitment and differentiation (Nakashima et al. 2002). Osterix expression suggests that the cells are at a more committed stage of osteogenic differentiation. Runx2 expression might have been due in part to the presence of FGF2 during the enrichment phase, as FGF2 is known to induce Runx2 expression (Montero et al. 2000).

Although Runx2 and Osterix expression are important regulators in the commitment of MSCs towards bone differentiation, they are also expressed in chondrocytes. Runx2 and Osterix expression was shown to be important in the late stages of chondrocyte differentiation and hypertrophy (Inada et al. 1999; Kim et al. 1999; Komori 2005; Oh et al. 2012). Therefore, their expression in phase II supports the presence of a chondro-osteoprogenitor population.

There are two additional possibilities that could contribute to enriching for the differentiation of a chondrogenic population during the mesoderm enrichment phase. First, the culture model used, rely on culturing cells in a suspension form. This might have contributed to directing cells towards chondrogenesis by mimicking the condensation stage, which is an essential step during cartilage development (Hall & Miyake 2000). Second, in the mesoderm enrichment phase, cultures were supplemented with FGF2 for 3 days. FGF2 signalling is known to be essential during skeletal development, particularly in limb bud development (Savage et al. 1993; Fallon et al. 1994; Yu & Ornitz 2008). In support of the differentiation of mesoderm towards limb bud mesenchyme, was the expression of Prx1 and a preliminary experiment conducted to culture cells in FGF2 only in phase III, showed that the chondrocyte nodules generated had a morphology that resembled chondrocyte nodules produced in limb bud micromass cultures (Sui et al. 2003) (data not shown). These results suggest that the differentiation culture conditions may resemble the signals characteristic of limb bud chondrocyte differentiation.

In conclusion, the mesoderm enrichment phase (phase II) differentiated the ESC-derived mesoderm towards a bi-potential chondro-osteoprogenitor population.

3.4.3 ROCK inhibition differentially regulates sub-mesodermal and chondro-osteoprogenitor populations at the mesoderm enrichment phase (phase II)

Investigating the role of ROCK inhibition at an intermediate developmental stage, such as the mesoderm enrichment phase in ESCs is done here for the first time. Inhibition of ROCK signalling at phase II upregulated the expression of paraxial markers (except Pax1), suggesting differentiation of cells towards the axial bone lineage. In addition, Flk-1 (a lateral plate mesoderm marker) and Prx1 (a limb bud marker) were also upregulated upon ROCK inhibition with no effect on the other lateral plate markers tested (Mesp1 and Foxf1a), which represent appendicular bone differentiation. This might suggest that ROCK inhibition directed the differentiation towards somite/sclerotome more than somatic/limb bud mesoderm development. Interestingly, in comparison to ROCK untreated cultures (7d-Y), ROCK inhibition showed a general downregulation of all mesoderm markers, suggesting that ROCK inhibition differentially regulated both paraxial and lateral plate mesoderm sub-mesoderm markers at the mesoderm enrichment phase (Phase II: 4-7d).

Moreover, the ROCK inhibition directed the differentiation of ESC-derived mesoderm towards a chondro-osteoprogenitor population by unregulating the expression of Sox9 and Sox5 as well as Runx2 and Osx. Interestingly, however, ROCK inhibition (7d+Y), in comparison to ROCK unexposed cultures (7d-Y), led to downregulation of Sox9 and Osx expression. In addition, Sox9 was weakly expressed at the protein level compared to Y27632-untreated cultures, which correlated with the mRNA expression. The weak expression may suggest reduced Sox9 activity upon ROCK inhibition. However, quantification of Sox9 activity is required to confirm this observation on a larger sample size. The complex effects of ROCK inhibition on Sox9 activity has been reported in other studies, with differential effects being explained by the effects of culture model used, 2 verses 3-dimensional cultures (Woods et al. 2005; Woods & Beier 2006). Woods et al. (2006) reported that even when Sox9 expression was upregulated, the expression of Sox9 down stream targets, Col II and aggrecan was downregulated in micromass culture, linked to the reduction in Sox5 and Sox6 expressions. In this project, the cells were cultured in a suspension form for 3 days.

ROCK inhibition, (7d+Y vs 7d-Y), upregulated Sox5 expression and had no effect on Runx2 expression. These results might indicate that ROCK inhibition signalling primes the differentiation towards cartilage over bone lineage.

Other studies reported a role of RhoA/ROCK in the commitment of hMSC to the osteogenic lineage. McBeath et al., (2004) showed by using micropatterned surfaces that activation of Rho/ROCK signalling stimulated osteogenesis and upregulated the osteogenic markers Runx2 and ALP. In addition, cells infected with an adenovirus encoding constitutively active RhoA stimulated osteogenesis in unspread cells while infection with adenovirus encoding constitutively active ROCK induced the osteogenesis irrespective of cell shape. On the contrary, Meyers et al. (2005) reported using a modeled microgravity culture model, that transfecting hMSCs with an adenovirus containing constitutively-active RhoA inhibited osteogenic differentiation. However, this study did not investigate if there is a role for the downstream effector, ROCK, on osteoblast differentiation. Interestingly, in the context of haematopoietic differentiation, Chen et al (Abstract published in ISSCR, 2013), demonstrated that ROCK inhibition using Y27632 in ESCs harbouring a Flk1 reporter gene, is a strong inducer of Flk-1 expressing cells and upregulated posterior primitive streak and haematopoietic markers, suggesting that ROCK signalling can modulate mesodermal cell commitment towards the haematopoietic lineage.

In summary, ROCK inhibition in the mesoderm enrichment phase (phase II) proved to differentially regulate the differentiation of ESC-derived mesoderm towards a distinctive chondro-osteoprogenitor population. In addition, ROCK inhibition might be a key in modulating chondrogenic over osteogenic potential when compared to ROCK unexposed cultures. Further kinetic gene expression analysis of cartilage and bone markers would be helpful in understanding the maturity stage of the cells before differentiating them in phase III as well as the downstream signalling factors controlling cartilage and bone differentiation at the mesoderm enrichment phase (phase II). In addition, quantification of the percentage of progenitors expressing cartilage and bone specific markers by FACS, or using cells that are GPF-labeled will provide further data on the efficiency of the differentiation protocol. The effect of ROCK inhibition on the functional potential *in vitro and in vivo* will be interesting to explore since the cells proved to be primed towards the chondrogenic and osteogenic lineages at

the molecular level. This will be investigated further in the next chapters (chapter 4 for *in vitro* cartilage differentiation, chapter 6 for *in vitro* bone differentiation, and chapter 7 for *in vivo* differentiation).

Chapter 4 Effect of phase-specific ROCK inhibition on the chondrogenic differentiation of ESC-derived mesoderm

4.1 Introduction

Chondrocyte differentiation undergoes a multi–stage cellular process that involves proliferation, differentiation and ends with maturation. During embryonic development of endochondral bone, cartilage formation includes several stages, whereby mesenchymal cells undergo condensation followed by chondrocyte differentiation (chondrogenesis), which is then replaced by osteoblasts (osteogenesis) (Hall & Miyake 2000; Olsen et al. 2000; Goldring et al. 2006; Zhang et al. 2009; Lefebvre & Bhattaram 2010). Chondroctyes are the only cellular component in cartilage (Pitsillides & Beier 2011). Chondrogenesis is a multistep process controlled by morphological and molecular changes (chapter 1, section1.4.11.5).

Cartilage is composed of chondrocytes, specific proteoglycans such as aggrecan and characteristic exracellular matrix proteins. These components are altered during the process of chondrocyte differentiation and maturation. Differentiated chondrocytes are characterised by a round or polygonal morphology that secret collagen type II (herein, Col II) and aggrecan (herein, Acan), while hypertrophic chondrocytes are enlarged cells and secret collagen type X (herein, Col X) (Hall & Miyake 2000; Hartmann 2009; Raggatt & Partridge 2010; Keller & Nieden 2011; Pitsillides & Beier 2011).

Small GTPAse signalling has been shown to influence chondrogenesis (Beier & Loeser 2010) in terms of lineage commitment (Gao et al. 2010), chondrogenic gene marker expression, differentiation and maturation (Wang, Woods, Sabari, Pagnotta, L. Stanton, et al. 2004; Woods et al. 2005; Woods & Beier 2006; Woods, Wang, Dupuis, et al. 2007; Kumar & Lassar 2009; Appleton et al. 2010; Haudenschild et al. 2010; Kim et al. 2012; Dy et al. 2012; Furumatsu et al. 2013). Specifically, the role of Rho/ROCK signalling in chondrogenesis appears to be complex. Several reports have suggested a positive role for Rho/ROCK signalling in chondrogenic commitment and differentiation (Woods et al. 2005; Tew & Hardingham 2006; Woods & Beier 2006),

While others have shown the contrary, in particular, using the Y27632 chemical inhibitor (Woods & Beier 2006). This may be due to several possibilities, such as culture conditions (e.g. 2- versus 3-dimensional cultures), cell origin and the stage of cell development during the differentiation process. Thus, the exact

mechanism of ROCK signalling in regulating chondrogenic development is not yet clear and unravelling the complexity of this signalling pathway in chondrogenic differentiation has been an interest in the field of musculoskeletal diseases such as osteoarthritis. Therefore, establishing an ES/EB, step-wise, mESC mesoderm differentiation model system (discussed in chapter 3) allows for a controlled and defined method for dissecting and further understanding of the molecular events occurring during development of cartilage, in particular, specification and differentiation. Thus, this chapter will investigate the effect of ROCK inhibition, specifically at two developmental stages: the mesoderm enrichment phase (phase II) and the differentiation phase (phase III).

4.2 Methods

The 3 phase ES/EB differentiation model system (discussed in chapter 3, Fig.3.1) was used, which included mesoderm induction (phase I) followed by the mesoderm enrichment phase (phase II) then a monolayer differentiation phase (phase III) (details in chapter 2, Fig.2.1). In the differentiation (phase III), cells were re-plated as single cells at a density of 10,000 cells/6mm well in a monolayer culture containing chondrogenic media supplemented with 100ng/ml BMP4 and 10ng/ml FGF2.

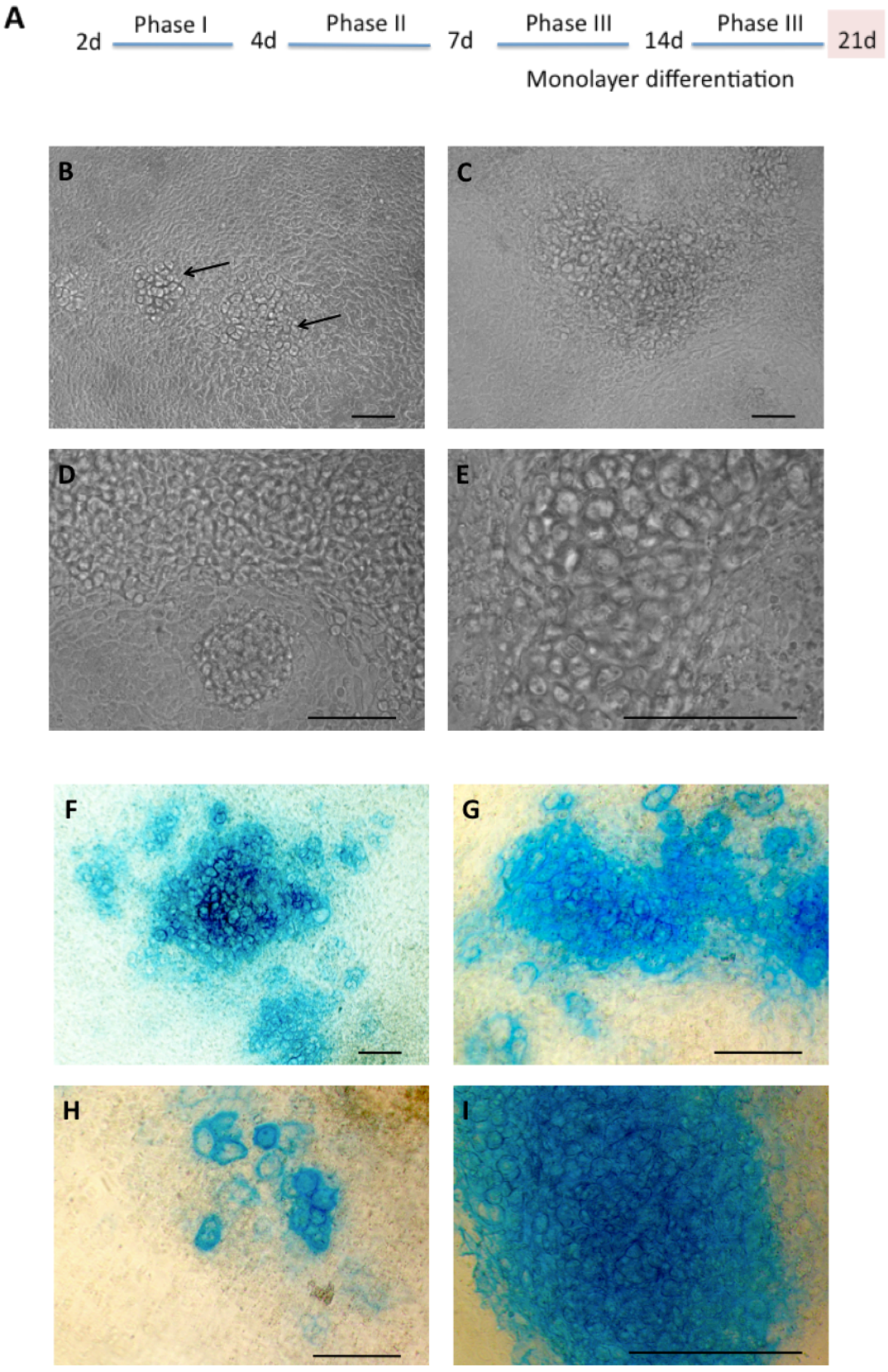
To test the effects of ROCK inhibition, Y27632 was added at specific time points during differentiation, as will be shown in each section. Cultures were fixed after 2 weeks. Analysis was undertaken by monitoring the morphological changes of the cells during the culture period and quantification of Alcian blue staining of chondrocyte nodules, an indicator of sulphated glycosaminoglycans, which is an established marker of chondrogenesis. Gene expression analysis of chondrogenic markers was performed by qPCR at several time points during differentiation, specifically at 4, 7, 14 and 21d.

4.3 Results

4.3.1 Chondrogenic differentiation of ESC–derived mesoderm

In chapter 3, molecular analysis showed that both Sox9 and Sox5 were upregulated after culturing cells in FGF2 for 3 days in the mesoderm enrichment phase (phase II) (chapter 3, Fig. 3.10). Hence, during the monolayer differentiation phase (phase III), the chondrogenic potential of the cells generated from the mesoderm enrichment phase (phase II) was examined by differentiating them in chondrogenic media (table 2.3) as shown in the timeline (Fig. 4.1A).

Firstly, analysis of morphological changes revealed that chondrocyte nodule formation appeared after 1 week of culture in chondrogenic media. After another week in culture, chondrocyte cells developed into 3-dimensional, cobblestone-like chondrocyte nodules (Fig. 4.1B-E). The chondrocyte nodules produced an extracellular matrix proteoglycan that stained positively with Alcian blue (Fig. 4.1F-I). Further evidence confirming the presence of chondrocytes was gained by investigating the expression of type II collagen protein. Immunofluorescence analysis demonstrated that differentiated chondrocyte nodules were also positively stained with a collagen type II antibody (Fig. 4.1J,K).


A
2d
Phase I
4d
Phase II
7d
Phase III
14d
Phase III
21d
Monolayer differentiation
B
C
D
E
F
G
H
I

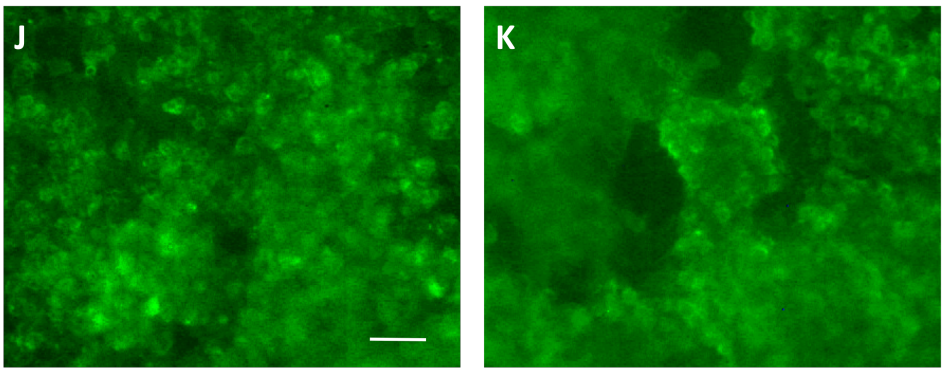


Figure 4.1: Chondrogenic differentiation of ESCs-derived mesoderm in the monolayer differentiation phase (phase III).

A) A differentiation scheme showing the timing of chondrogenic differentiation. Cells were cultured in chondrogenic media (FGF2 (10 ng/ml), BMP4 (100ng/ml)). Monolayer cultures were fixed after 2 weeks for analysis. B-E) Phase-contrast and bright-field images of monolayer cultures showing typical cartilage nodules with rounded chondrocyte morphology. F-I) Alcian blue staining of chondrocyte nodules showing chondrocyte cells surrounded by an Alcian blue positive ECM. J,K) Collagen type II immunofluorescence staining of chondrocyte nodules. All images are from 21d cultures and from representative wells (n= 11 experiments). Scale bars: 10um.

Molecular analysis of the cultures after 2 weeks of differentiation (7-21d) (Fig. 4.2A) showed an upregulation of Sox9 expression at 14d compared to 7d cultures, which was then downregulated at 21d, the end of the differentiation period compared to 14d cultures. On the other hand, no differences were found in Col II expression in 14d and 7d cultures, whilst its expression was then upregulated at the end of the differentiation at 21d compared to 14d cultures. The hypertrophic chondrocyte marker type X collagen (Col X) was expressed, however, at a negligible level compared to Sox9 and Col II expression (Fig. 4.2B). Col X expression was slightly upregulated at 14d compared to 7d cultures and then downregulated at 21d of differentiation compared to 14d cultures. Collectively, the results suggest that mesoderm-derived ESCs can differentiate efficiently towards the chondrogenic lineage.

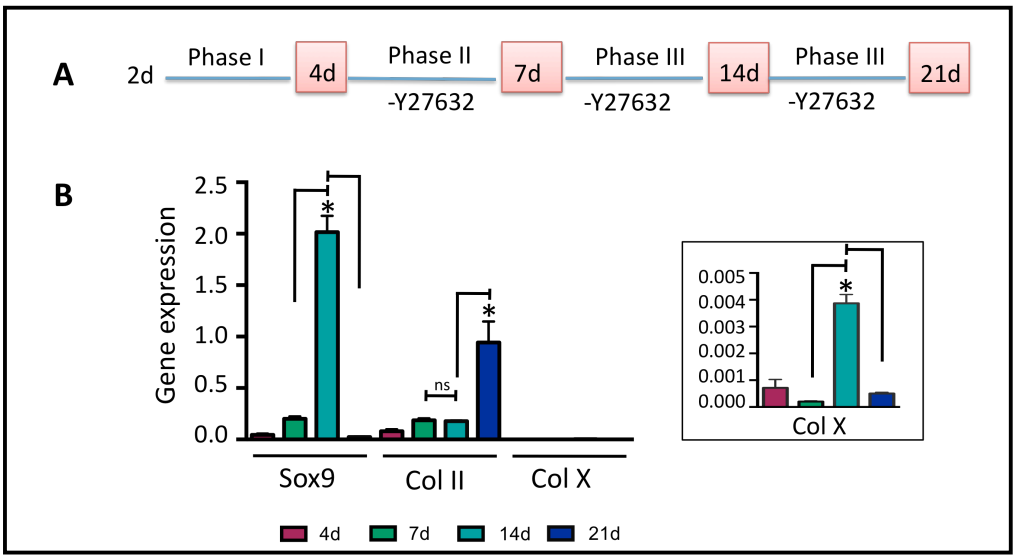


Figure 4.2: Expression of chondrogenic markers after 2 weeks in the monolayer differentiation phase (phase III).

A) The differentiation scheme showing in pink boxes the time points at which RNA was extracted during the differentiation at 4, 7, 14 and 21d. B) Quantitative PCR analysis of chondrogenic markers gene expression (Sox9, Col II, and Col X). The insert shows a magnification of Col X expression. Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (paired student's t-test; * $p < 0.05$, 7d vs 14d and 14d vs 21d).

4.3.2 Effect of ROCK inhibition during the mesoderm enrichment phase (phase II) on chondrogenic differentiation

In chapter 3, Y27632 treatment during the mesoderm enrichment phase (phase II) illustrated a differential effect on the expression of the chondrogenic transcription factors (Sox9 and Sox5) compared to the Y27632-untreated cultures (Fig.3.13). To further investigate the functional potential of the generated cells, cells were cultured in the absence and presence of Y27632 in phase II, followed by culture in chondrogenic media in the absence of Y27632 for 2 further weeks (Fig. 4.3A). Analysis of the morphological changes in culture revealed that chondrocyte nodules were observed in both Y27632-treated and untreated monolayer cultures after 1 week in chondrogenic media, and this was clearly evident following Alcian blue staining (Fig. 4.3B). However, quantification of nodule formation following Alcian blue staining showed a 3-fold increase in the percentage of Alcian blue positive nodules in Y27632-treated cultures,

compared to untreated cultures (Fig. 4.3C). This increase was verified by quantification of the Alcian blue staining following solubilisation (Fig. 4.3D). These results suggest that short-term inhibition of ROCK signalling in phase II (4-7d) induced chondrogenic differentiation afterwards in phase III (7-21d) in the absence of further ROCK inhibition.

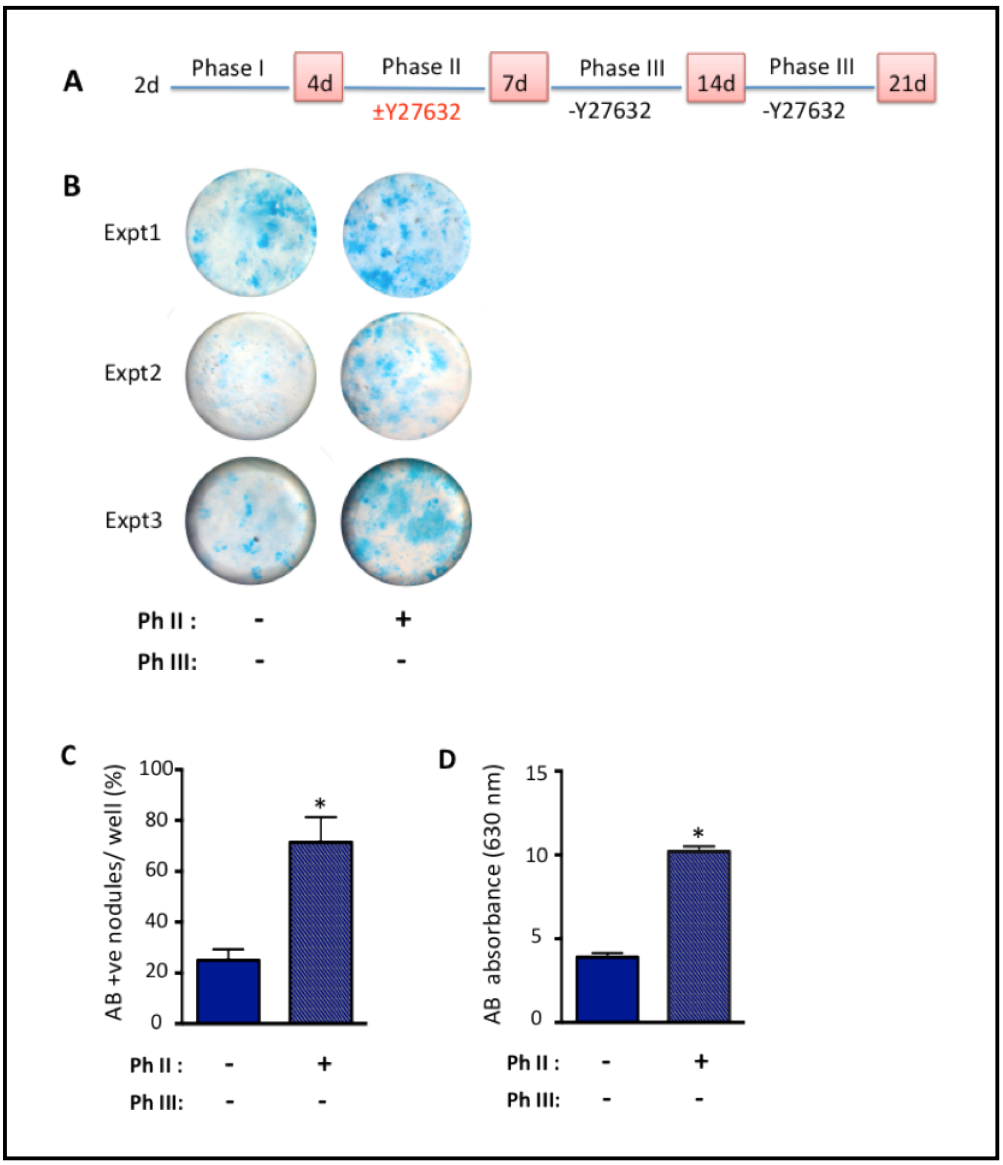


Figure 4.3: The effect of temporal ROCK inhibition in phase II on chondrogenic differentiation of ESC-derived mesoderm.

A) A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. In phase III, cells were cultured in chondrogenic media for 2 weeks then fixed for analysis. B) Representative images of Alcian blue stained wells from 3 independent experiments. C) The percentage of Alcian blue-positive nodule area was counted manually using the point-counting method. D) Alcian blue staining was solubilised and quantified by measuring absorbance at 630 nm. Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (paired student's t-test; * $p < 0.05$).

Subsequently, molecular analysis of (14d) cultures (Fig. 4.4B, green bars) confirmed the cellular effects demonstrated with Y27632 (+Y) treatment through the upregulation of the expression of Sox9 and Acan compared to the untreated cultures (-Y). Meanwhile, there were no differences in the expression of either Col II or Col X in Y27632-treated cultures (+Y), compared to the untreated cultures (-Y) (Fig. 4.4B, green bars). On the other hand, analysis of (21d) cultures (Fig. 4.4B, blue bars), showed that Y27632 treatment (+Y) upregulated all four chondrogenic genes in comparison to the untreated cultures (-Y).

Moreover, analysis of Y27632-untreated cultures (-Y) at (21d) revealed that all genes were downregulated by the end of the differentiation phase compared to (14d) cultures (Fig. 4.4B, -Y14d vs -Y21d). Interestingly, however, Col II expression at 21d, in the absence of Y27632 (-Y), showed a substantial increase compared to 14d cultures (Fig. 4.4B, -Y14d vs -Y21d). Similarly, Y27632-treatment also showed similar pattern (Fig. 4.4B, +Y14d vs +Y21d) These results suggest that ROCK signalling inhibition at the mesoderm enrichment phase (phase II) enhanced chondrogenesis at the mRNA level. In addition, ROCK signalling inhibition in phase II seems to direct the chondrogenic differentiation towards hypertrophy.

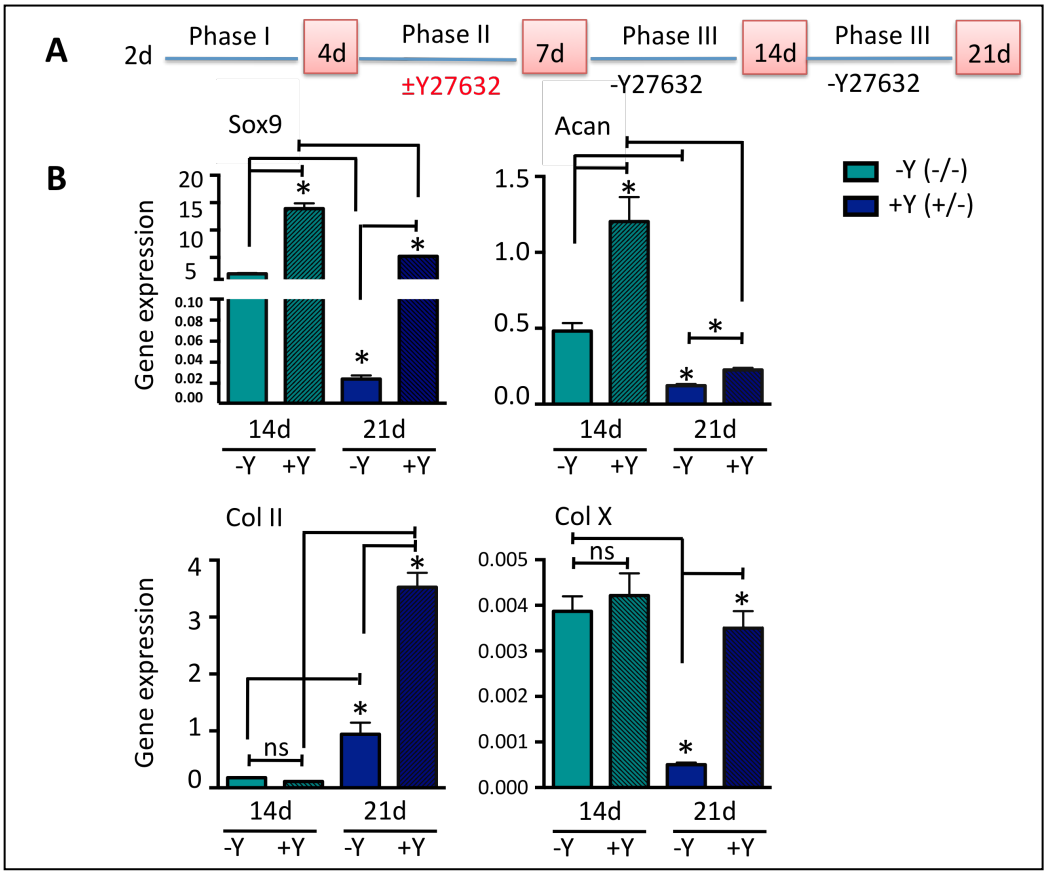


Figure 4.4: The effect of ROCK inhibition in phase II on the expression of chondrogenic markers in phase III.

A) A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. In phase III of differentiation, RNA was extracted at 2 time points during the differentiation; at 14d (after 1 week in the differentiation phase) and at 21d (after 2 weeks in the differentiation phase). ±Y represents treatment with Y27632 during phase II. B) Quantitative PCR analysis of chondrogenic marker expression (Sox9, Acan, Col II, and Col X). Data indicate mean ± SD (triplicates) (n=2 for 14d) and (n=3 for 21d)(paired student's t-test; * $p < 0.05$).

4.3.3 Effect of ROCK inhibition in both phases II and III on chondrogenic differentiation

Following the results from the previous section, further investigation was conducted on whether inhibiting ROCK continuously in both phases II and III (4-21d) would influence chondrogenic differentiation. For this reason, cells in phase II were cultured in both the absence and presence of Y27632, followed by culture in chondrogenic media, this time in the presence of Y27632 for a further 2 weeks (Fig. 4.5A). Quantification of Alcian blue staining at this stage demonstrated a 7.2-fold increase in the percentage of Alcian blue positive

nodules, and Alcian blue solubilisation quantification confirmed this increase (Fig. 4.5B-D). This indicates that the long-term inhibition of ROCK signalling during both the mesoderm enrichment and differentiation phases (4-21d), not only induced but also enhanced the chondrogenic differentiation of mesoderm derived ESCs compared to short-term ROCK inhibition (7-21d).

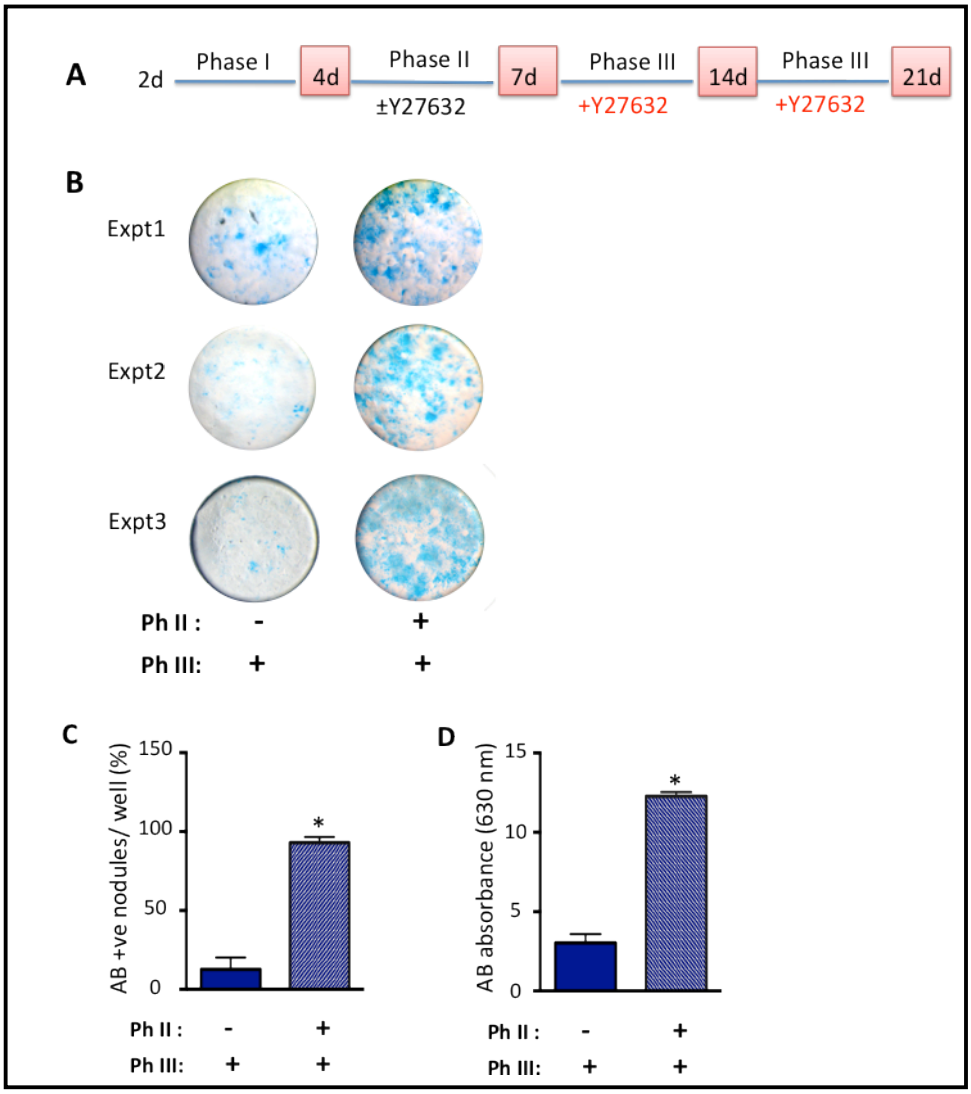


Figure 4.5: The effect of continuous Y27632 exposure in phases II and III on chondrocyte nodule quantification and GAG synthesis.

A) A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. In phase III, cells were cultured in chondrogenic media for 2 weeks then fixed for analysis. B) Representative images of Alcian blue stained wells from 3-5 independent experiments. C) The percentage of Alcian blue-positive nodule area was counted manually using the point-counting method. D) Alcian blue staining was then solubilised and quantified by measuring absorbance at 630 nm. Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (paired student's t-test; * $p < 0.05$).

Quantitative PCR analysis was then performed for chondrogenic markers expression at (14d) cultures and (21d) cultures from temporal (-/+) and continuous Y27632 treatment (+/+) as shown in the time line (Fig. 4.6A), in this section for the simplicity of presentation, temporal (7-21d) cultures will be defined as (-Y) and continues cultures will be defined as (+Y).

The results showed at 14d of differentiation, that Y27632 treatment upregulated Sox9, Acan and Col X expression with no effect on Col II expression in comparison to Y27632-untreated cultures (Fig. 4.6B, green bars, -Y14d vs +Y14d). Interestingly, qPCR analysis of 21d cultures showed that Y27632 treatment upregulated both Col II and Col X with no effect Sox9 expression and a slight, but significant downregulation in Acan expression compared to Y27632-untreated cultures (Fig. 4.6B, blue bars, -Y21d vs +Y21d). Analysis of Y27632-untreated cultures revealed a downregulation in Sox9 expression with no effect on Acan, Col II, and Col X expressions by the end of the differentiation phase (21d) compared to (14d) cultures (Fig. 4.6B, -Y14d vs-Y21d). While upon Y27632 addition, no effect was shown on Sox9 and Acan expression by the end of the differentiation phase, however, Col II Col X expression was upregulated compared to 14d cultures (Fig. 4.6B, +Y14d vs +Y21d).

These results supported the observation that long-term exposure to ROCK inhibition in both phases II and III (4-21d) stimulated the differentiation of a distinctive chondrogenic population with an enhanced hypertrophic chondrocyte differentiation potential at the mRNA level in comparison to short-term ROCK inhibition exposure in phase III only.

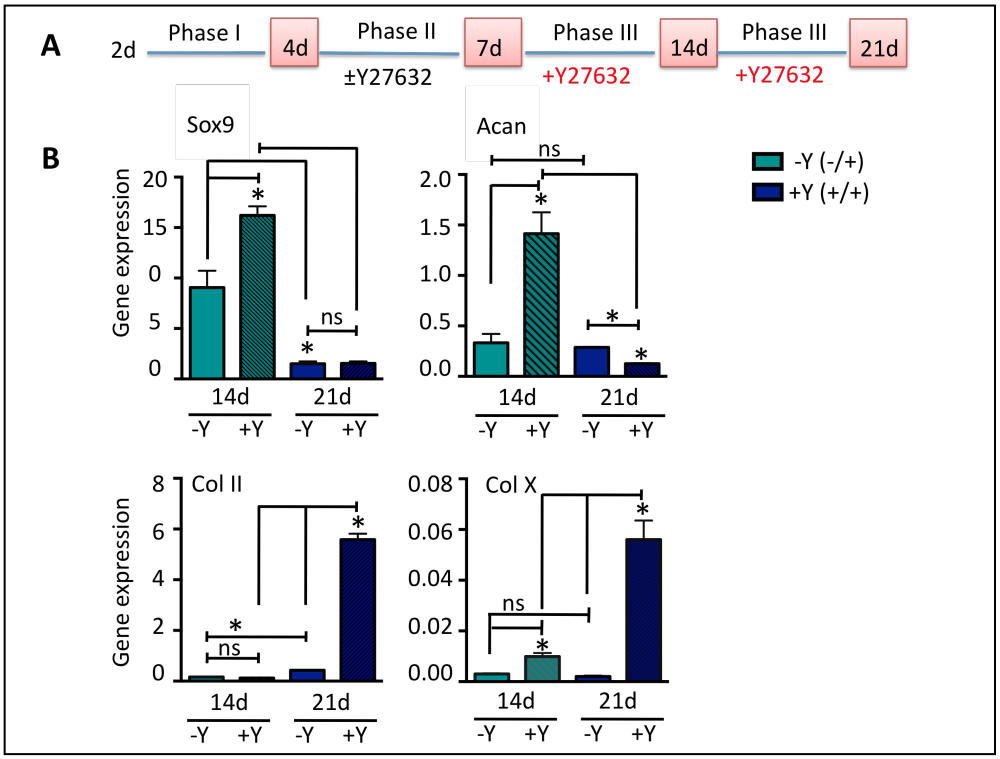


Figure 4.6: The effect of continuous Y27632 exposure in phases II and III on chondrogenic marker expression

A) A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. In phase III of differentiation, RNA was extracted at 2 time points during the differentiation; at 14d (after 1 week in the differentiation phase) and at 21d (after 2 weeks in the differentiation phase). ±Y represents treatment with Y27632 during phase II. B) Quantitative PCR analysis of chondrogenic markers expression (Sox9, Acan, Col II, and Col X). Data indicate mean ± SD (triplicates) (n=2 for 14d) and (n=3 for 21d)(paired student's t-test; * $p < 0.05$).

Summarising the effects of ROCK inhibition across the different Y27632 treatment protocols (Fig. 4.7A), the results show that only a 3-day pulse of Y27632 treatment during the mesoderm enrichment phase (phase II: 4-7d), increased Col II and Col x expression by a 3-fold and 83-fold, respectively (Fig. 4.7B,C; -/- vs +/-). Moreover, continuous exposure to Y27632 during both the mesoderm enrichment and differentiation phases (phase II and III: 4-21d) showed a 4-fold and 250-fold increase in both genes, suggesting further enhanced cartilage differentiation (Fig. 4.7B,C; -/- vs +/+). However, temporal Y27632 treatment during the differentiation phase III alone (7-21d) showed a 2-fold reduction in Col II expression, suggesting that late exposure was not sufficient to stimulate chondrogenesis and may even inhibit it (Fig. 4.7B,C; -/- vs -/+). In parallel, Col X expression was increased by 35-fold, but at very low

levels. Taken together, the findings reveal that inhibition of ROCK starting from the early mesodermal stage is sufficient to stimulate chondrogenic differentiation of ESC derived mesoderm. Therefore, ROCK inhibition in phase II is the key in inducing chondrogenesis and continous ROCK inhibition enhaned the differentiation and maturation.

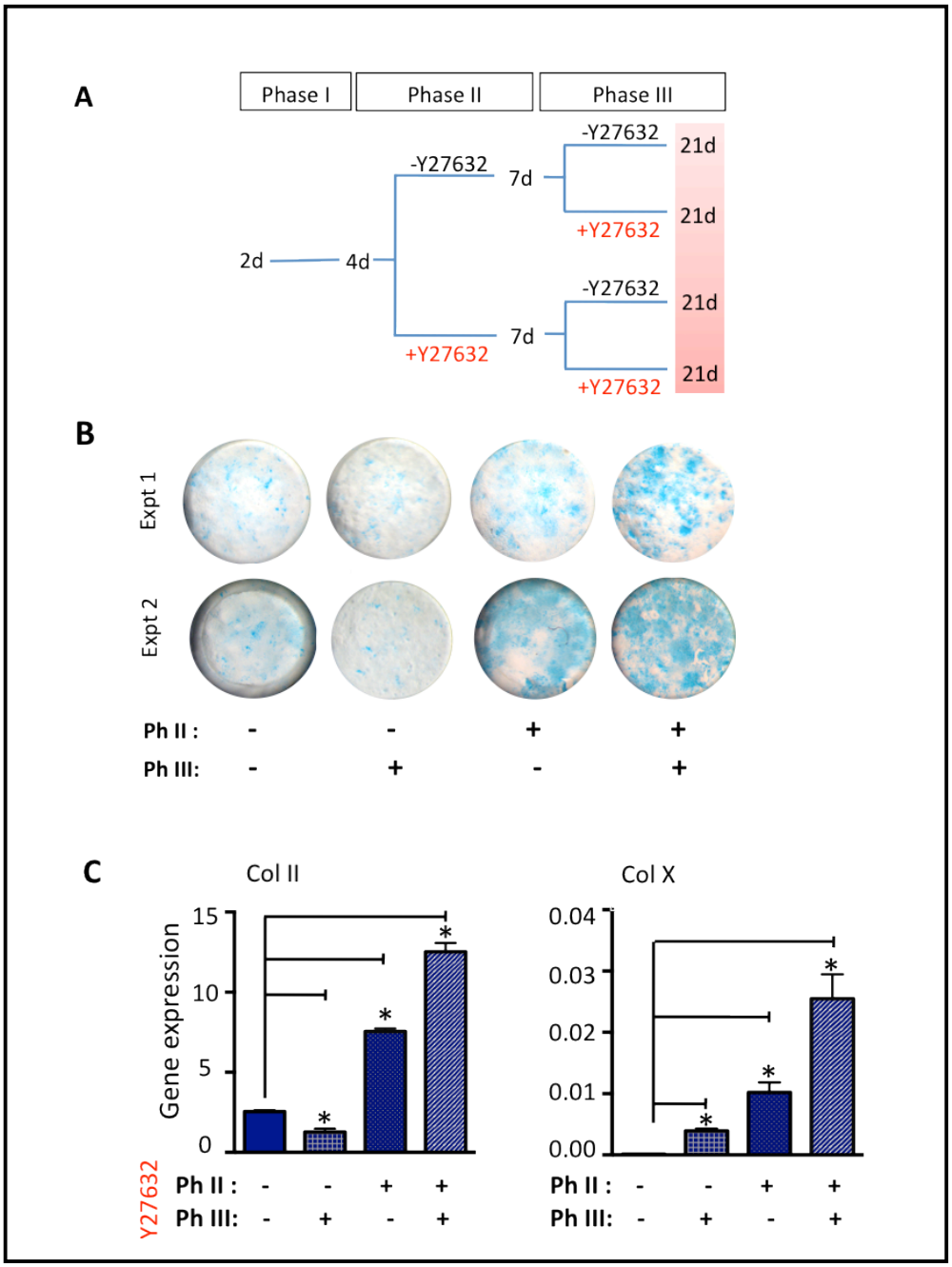


Figure 4.7: Summary of the effects of temporal ROCK inhibition on Col II and Col X gene expression.

A) A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. B) Representative images of Alcian blue stained wells from 2 independent experiments. C) RNA was extracted at 21d for analysis of chondrogenic marker expression (Col II, Col X) using qPCR. Data indicate mean ± SD (triplicates) from an experiment other than previously shown (one-way ANOVA test; * $p < 0.05$).

4.4 Discussion

This chapter has demonstrated, in a serum free, 3-phase ES/EB culture model, an effective directed differentiation of ESC-derived mesoderm towards chondrocytes. In addition, the chondrgenic effect of ROCK signalling inhibtion proved to be a phase-specific effect. Temporal ROCK inhbition (+/-) during the mesoderm enrichment phase was shown to be the key in driving the chondrogenic differentiation of ESC-derived mesoderm. Furthermore, continous ROCK inhibition in both the mesoderm enrichment phase and differentiation phase (+/+) proved to enhance the chondrogenic differentiation towards hypertrophic cartilage. Therefore, a short-term ROCK inhibition, specifically at the mesoderm enrchment phase (+/-) may have a distinctive role in influencing cartilage specification, differentiation, and maturation.

The functional consequences for chondrogenesis were analysed systematically here by testing the effects of short, temporal Y27632 exposure in phase II only (+/-), followed by phase III chondrogenic differentiation in the absence or presence of the ROCK inhibitor. This will be discussed further in the following sections.

As mentioned in the introduction, the inhibition of Rho/ROCK signalling showed controversial results in terms of its role in chondrogenic differentiation, explained by the possibility of a cell context dependent effect (Woods et al. 2005; Woods & Beier 2006). This may be due to several possibilities, such as culture conditions (e.g. 2- versus 3-dimensional cultures), cell origin, and the stage of cell development during the differentiation process. It was proved that by using our 3-phase differentiation culture model, the role of ROCK inhibition on chondrogenic differentiation could be addressed through a defined and controlled approach, by analysing the ROCK signalling inhibition effect in two developmental stages: mesoderm enrichment (phase II) and the differentiation phase (phase III), which will be further discussed in the following sections.

4.4.1 Temporal ROCK inhibition (4-7d)

A short pre-exposure to ROCK inhibitor during the mesoderm enrichment phase (+/-) led to the induction of chondrogenesis later in the differentiation phase (Phase III) in the absence of further ROCK inhibition. The significant, 3-fold increase in the percentage of Alcian blue-positive nodules suggested that exposing cells to a short pulse of ROCK inhibitor at the mesoderm enrichment phase (+/-) might have targeted early chondrogenic precursors. In addition, Alcian blue dye extraction and measurement of absorbance confirmed the sulphated glycosaminoglycan synthesis.

Additionally, as described in the previous chapter (Chapter 3), expression profiling during the mesoderm enrichment phase (Phase II) proved that the cells generated were primed towards chondrogenesis. The 3-day FGF2 treatment enriched the cell population towards paraxial and lateral plate mesoderm as well as for chondrogenesis as measured by gene expression, and ROCK inhibition modulated the expression profile of both populations. The results showed that both pre-chondrogenic mesoderm markers such as Nkx3.2, and Prx1 (chapter 3) (Leussink et al. 1995; Rodrigo 2003) and Sox genes (Sox9 and Sox5) expression were upregulated prior to the differentiation phase (phase III).

Several studies demonstrated the chondrogenic effect of ROCK signalling inhibition in different cell context. Woods et al. (2005) reported that ROCK inhibition, using Y27632, showed round cell morphology, increased glycosaminoglycan synthesis, but did not increase the number of condensed Alcian blue positive nodules in limb micromass cultures and primary cell monolayer cultures. In our culture model, the actual number of nodules was not counted individually as this could not be done in an unbiased way. Instead the percentage of Alcian blue positive area was quantified according to clustering of the nodules as they reached the end of the differentiation. However, it would be interesting to quantify the effect of ROCK inhibition on chondrogenic precursors number during the differentiation period. In addition, Tew and Hardingham (2006) reported that ROCK signalling prevented dedifferentiation of articular chondrocytes phenotype when cells cultured in a monolayer form.

Furthermore, using a similar ESC culture system, Craft et al (2013) reported the differentiation of mesoderm-derived chondrocytes whereby the mesoderm induction media was supplemented with ROCK inhibitor Y27632.

In this project, the presence of ROCK inhibitor-sensitive cells generated from the mesoderm enrichment phase (phase II) was further evident at the molecular level when cultures were analysed at the differentiation phase (Phase III), showing an upregulation of chondrogenic marker expression. The analysis of one-week and two-weeks cultures expressed Sox9 and Acan, suggesting the existence of chondrogenic progenitors at this stage of differentiation that responded positively to ROCK inhibition. The molecular profile at 21d cultures analysis showed the upregulation of Sox9 and Col II expression in the ROCK inhibitor treated cultures as well as Col X, but at very low levels. Therefore, suggesting the advancement in the maturation of the cultures under the influence of ROCK inhibition.

Similarly, several studies in mouse (Woods et al. 2005; Woods & Beier 2006) and human (Haudenschild et al. 2010) cultures have reported that ROCK inhibition stimulates Sox9 expression as well as downstream targets, Col II and aggrecan. In the context of human articular cartilage, ROCK inhibition (Y27632) showed to activate Sox9 expression (Tew & Hardingham 2006). However, differences in the cell culture model showed to influence down stream targets of chondrogenic lineage even in the expression of Sox9 (Woods & Beier 2006). Furthermore, Woods et al. (2006) reported that Col II and aggrecan expression was upregulated in ATDC5 cell line culture and primary cell monolayer cultures while downregulated in micromass cultures.

Surprisingly, short-term exposure to ROCK inhibition, this time in phase III only (7-21d), had no effect on chondrocyte differentiation and maybe even inhibited it slightly. The expression of Col II and Col X was very low in comparison to their expression when ROCK was inhibited only in phase II (4-7d) or inhibited in both phases II and III (4-21d). This effect may suggest that exposing cells to the ROCK inhibitor at phase III may be too late to stimulate the ROCK-sensitive populations or chondrogenic progenitors compared to exposing the cells to the ROCK inhibitor in phase II. This could be explained by the expression analysis of the mesoderm enrichment phase (phase II) (chapter 3), which showed that ROCK inhibitor-untreated cultures (7d-Y) expressed higher transcript levels of

mesoderm gene markers as well as cartilage and bone gene markers compared to ROCK inhibitor-treated cultures (7d+Y). Therefore, suggesting that ROCK-inhibited cultures might be at a different stage of mesoderm development compared to ROCK treated cultures. In conclusion, under the culture conditions used in this project, temporal ROCK inhibition in phase III only (7-21d) is not recommended for further studying of chondrogenesis.

4.4.2 Continuous ROCK inhibition (4-21d)

In contrast to the short-term effects of ROCK inhibition in the mesoderm enrichment phase (Phase II: 4-7d) on chondrogenic differentiation, the current study also proved that continuous ROCK inhibition in both phases II and III not only induced chondrogenesis, but also enhanced it towards hypertrophy. This was evident by the significant 7.2-fold increase in Alcian blue-positive nodule area as well as in the upregulation in expression of Col II and Col X. Interestingly, the effect of ROCK inhibition on chondrocyte hypertrophy demonstrated under our culture conditions corresponded with that shown by Wang et al. (2004). It was demonstrated that ROCK inhibition accelerated hypertrophic differentiation of chondrocytes in both the ATDC5 cell line and primary chondrocytes micromass cultures. This was confirmed by upregulation in the expression of both Col X and BSP as well as an increase in both ALP activity and mineralisation of cultures.

Indeed, our serum-free culture model could be a good foundation for future study of chondrocyte differentiation, in particular, hypertrophy and chondrogenic maturation, especially on the effect of ROCK inhibition signalling. Therefore, further studies of chondrogenic differentiation using the 3-phase differentiation model with continuous ROCK inhibition could pave the way for further understanding of molecular mechanisms downstream of ROCK inhibition signalling, which may contribute to driving and regulating chondrocyte differentiation and expansion.

Comparing the effects of short-term exposure to ROCK inhibition, (+/-) or (-/+), to continuous exposure to ROCK inhibition (+/+) showed the greatest increase in both chondrocyte positive nodule areas in the expression of both Col II and Col X chondrogenic markers.

Indeed, the chondrogenic differentiation observed upon ROCK inhibition led to an efficient differentiation of chondrocytes covering up to ~95% of culture area. In human ESC cultures, Oldershaw et al. 2010 reported, in 3 cell lines, an efficient differentiation of chondrocyte that comprised 74-97% of Sox9 expressing cells, using a serum free, 3 stage differentiation model. However, they did not show functional differentiation of the generated cells. Therefore, in the current study it will be interesting to examine the percentage of Sox9 expressing cells in the cultures prior to the chondrogenic differentiation stage.

In summary, using the novel culture conditions and the 3-phase culture model, the results in this chapter proved for the first time a temporal effect of ROCK signalling on mesoderm-derived chondrogenesis by using ROCK inhibitor (Y27632). It was proven that pre-exposing cells to ROCK inhibition in the mesoderm enrichment phase (+/-) was the driving force in the induction of chondrogenesis, which was demonstrated by functional analysis in the differentiation phase (-/+). In addition, continuous exposure to ROCK inhibition during the mesoderm enrichment and differentiation phases (+/+) advanced the chondrogenic differentiation towards hypertrophy. Therefore, it will be interesting to examine the *in vivo* potential of the differentiated cells and their ability to generate a stable articular or growth plate-like cartilage phenotype. Thus, this protocol for cartilage differentiation could be then translated to human ESCs and iPSCs for the understanding of human diseases such as osteoarthritis and skeletal bone growth. The *in vivo* potential of the cells will be further discussed in chapter 7.

Chapter 5 The relationship between FGF2, BMP4 and GDF5 signalling in ROCK-dependent chondrogenic differentiation and maturation

5.1 Introduction

In this project, the findings presented in chapter 4 clearly demonstrated that phase-specific manipulation of ROCK signalling inhibition enhanced chondrogenic differentiation towards hypertrophy. Therefore, the results instigated further examination in two aspects of chondrocyte differentiation (i.e. hypertrophic and non-hypertrophic chondrocyte differentiation) and the role of ROCK signalling inhibition in this process. However, optimisation of the terminal differentiation media was considered first.

During embryonic development and postnatal life, most bone of the skeleton undergoes endochondral ossification, whereby a cartilage template is formed which is then replaced by bone (Hall & Miyake 2000; Olsen et al. 2000; Goldring et al. 2006; Mackie et al. 2008; Long & Ornitz 2013b). In the long bones of the limbs, such as the femur and humerus, chondrocytes undergo cycles of proliferation, hypertrophy and apoptosis. Chondrocytes closest to the end of long bones, proliferate (called resting chondrocytes). After that, they undergo accelerated proliferation and form characteristic columns of cells for longitudinal bone growth. Finally, cells reach a terminal differentiation phase prior to matrix calcification, at which hypertrophic chondrocytes develop, which are characterised by an increase in cell volume, secretion of the extracellular matrix protein, Col X, and expression of Matrix Metalloproteinase 13 (MMP13), ALP, Runx2 and Ihh. This provides the setting for blood vessel invasion and bone mineralisation (Inada et al. 1999; Long et al. 2001a; Jacenko et al. 2001; Kronenberg 2003; Lefebvre & Smits 2005; Goldring et al. 2006; Hidaka & Goldring 2008). Chondrocyte hypertrophy is a critical stage at which a transition occurs from chondrogenesis to osteogenesis (Goldring et al. 2006; N. S. Hwang et al. 2007; Solomon et al. 2008).

FGF signalling has been known for its mitogenic effect on different cell types, including chondrocytes (Solchaga et al. 2005; Chiou et al. 2006), however, it may also exhibit differential effects on chondrogenic proliferation and hypertrophy (Yoon et al. 2006; Sahni et al. 1999). FGF2, in particular, has been shown in several studies to stimulate proliferation while inhibiting hypertrophic differentiation in cultures of rat rib growth plate chondrocytes (Wroblewski & Edwall-Arvidsson 1995) and in micromass cultures of rabbit rib cells (Kato & Iwamoto 1990).

In contrast to chondrocytes in the growth plate, chondrocytes in the joint surfaces are required to maintain the integrity of articular cartilage and are characterised by specialised ECM consisting primarily of collagen type II (Col II), aggrecan (Acan) (Hidaka & Goldring 2008; Pitsillides & Beier 2011), and marked by the expression of various markers such as GDF5 (previously known as BMP14 in humans), which is a member of the TGFβ superfamily (Storm et al. 1994; Settle et al. 2003; Archer et al. 2003; Pacifici et al. 2006; Iwamoto et al. 2007; Khan et al. 2007) and lubricin (Prg4) (Flannery et al. 1999; Schumacher et al. 1999).

Recently, manipulation of BMP4 and GDF5 signalling has been shown to specify ESC-derived mesoderm towards hypertrophic and non-hypertrophic (articular-like) chondrocytes (Craft et al. 2013). Therefore, establishing an ES/EB, step-wise, mESC mesoderm differentiation model system (discussed in chapter 3) allows for a controlled and defined method for dissecting and further understanding the role of ROCK inhibition in directing chondrocyte differentiation towards distinct chondrocyte populations that are either hypertrophic or non-hypertrophic.

In the previous chapter (chapter 4), the finding demonstrated that a phase-specific manipulation of ROCK signalling inhibition enhanced chondrogenic differentiation towards hypertrophy; however, that occurred under the influence of both FGF2 and BMP4 signalling which were established as the basal differentiation conditions. In light of the potential opposing roles of FGF in regulating proliferation and differentiation, further inquiry was conducted into whether manipulating FGF2, in particular its duration of treatment under the culture conditions used in this project, could further influence the amount and type of cartilage formed *in vitro* (i.e. hypertrophic maturation) of ESC-derived mesoderm. In addition, it was investigated whether ROCK inhibition together with BMP4 or GDF5 signalling would influence ESC-derived mesoderm towards hypertrophic and non-hypertrophic (articular-like) chondrocytes.

5.2 Methods

The 3-phase ESC/EB differentiation model system as discussed in chapter 3 was also applied here. The same protocol was followed as was used for the chondrogenic differentiation studies described in chapter 4, whereby the chondrogenic media contained BMP4 (100ng/ml), FGF2 (10ng/ml). Exposure to FGF2 was either for the entire culture duration (herein, defined as continuous FGF2) or removed after 1 week of differentiation (at 14d) (herein, defined as temporal FGF2). Furthermore, under temporal FGF2 culture conditions, BMP4 was replaced by GDF5 (30ug/ml) as will be described later in this chapter.

To test the effects of ROCK inhibition, Y27632 (10mM) was added to the cultures, as will be shown in each section. Cultures were fixed after 2 weeks and analysis was undertaken by monitoring the morphological changes of cells in culture, quantification of Alcian blue positive nodule areas, Col X immunofluorescence staining as well as H&E with Alcian blue staining. Molecular analysis of chondrogenic marker gene expression Sox9, Col II, Col X and Prg4 was also performed by qPCR.

5.3 Results

5.3.1 The effect of ROCK inhibition and temporal FGF2 on chondrogenic differentiation and maturation

In chapter 4, it was demonstrated that phase-specific ROCK inhibition stimulated chondrogenesis and continuous exposure to ROCK inhibition enhanced this effect, as evidenced by an increase in the percentage of chondrogenic nodules and upregulation of chondrogenic gene expression, including Col X, the hypertrophic chondrocyte marker. All the former results were obtained from continuous use for 2 weeks of a chondrogenic medium containing FGF2 and BMP4. Recognising that long-term treatment with FGF2 might not provide the optimal balance between proliferation and differentiation led us to investigate the chondrogenic potential of cells when cultured in FGF2 alone, or in combination with BMP4 (Fig. 5.1A).

Alcian blue staining results showed, in comparison to control cultures (Fig. 5.1B,E,F), cultures treated with FGF2 only showed a significant reduction in

Alcian blue staining (Fig. 5.1C,G,H). In addition, BMP4 only cultures showed a visible increase in Alcian blue staining (Fig. 5.1D,I,J). These results suggest that FGF2 inhibits while BMP4 signalling stimulates chondrogenesis. However, observations of cells during the culture period showed that FGF2 cultures reached confluency earlier than BMP4 cultures (observational data), suggesting that FGF signalling is required for initial stage proliferation and/or expansion of chondrogenic precursors.

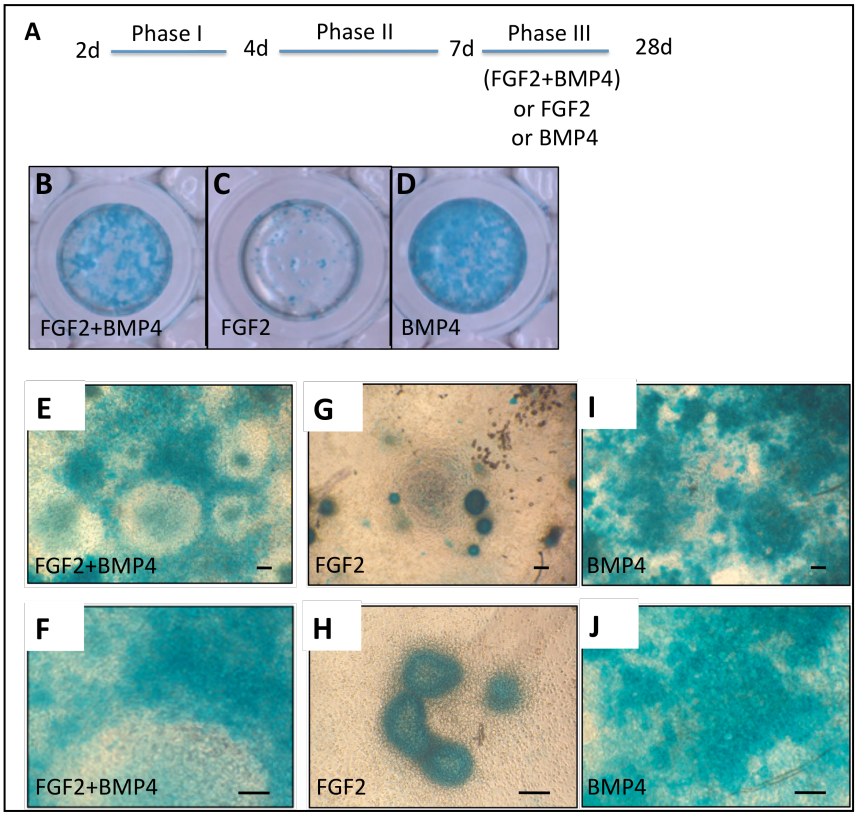


Figure 5.1: Effect of FGF2 and BMP4 either alone or in combination on chondrogenic differentiation.

A) A differentiation scheme showing the timing of factors additional to the culture media in phases II and III. In phase II, cells were cultured in FGF2 and Y27632. Then in phase III, cells were cultured in chondrogenic media containing FGF2 and BMP4 (either alone or in combination) in the further presence of Y27632 for 2 further weeks then fixed for analysis. B) Images of Alcian blue stained wells and (E&F) microscopic images from cultures treated with combined FGF2 and BMP4. C) Images of Alcian blue stained wells and (G&H) microscopic images from cultures treated with FGF2 only. D) Images of Alcian blue stained wells and (I&J) microscopic images from cultures treated with BMP4 only. All images are from one representative experiment of 2 independent experiments. Scale bars: 100 µm.

Based on the findings in Figure 5.1, demonstrating that FGF2 is required for initial stage proliferation and/or expansion of chondrogenic precursors and continuous treatment in FGF2 inhibited chondrogenic differentiation of ESC-derived mesoderm, it was investigated whether temporal exposure to FGF2 during the monolayer differentiation phase (phase III) could influence the amount and type of cartilage formed *in vitro*, and whether ROCK inhibition during the mesoderm enrichment phase (phase II: 4-7d) would influence this process.

As shown in Fig. 5.2A, in these experiments cells were first cultured in phase II in the presence or absence of Y27632 followed by differentiation (phase III) in the continued presence of Y27632. Cells were cultured in the continuous presence of FGF2 and BMP4 as control cultures (defined as continuous FGF2), and compared to cultures where FGF2 was removed after one week of culture (defined as temporal FGF2) (Fig.5.2A).

At the morphological level, in temporal FGF2 cultures, the Y27632-treated cultures showed large round cells that resembled hypertrophic chondrocytes compared to Y27632-untreated cultures (Fig. 5.2B&C). Moreover, the hypertrophic chondrocyte morphology was further confirmed by analysis of histological sections of fixed cultured tissue (Fig. 5.2D) and at the protein level by immunofluorescence for Col X (Fig. 5.2E).

Staining differentiated monolayer cultures with Alcian blue followed by quantification showed, under continuous FGF2 culture conditions, a 4-fold increase in GAG production in Y27632-treated cultures ($+Y_C$) compared to untreated cultures ($-Y_C$) (Fig. 5.2F&G, blue bars). Under temporal FGF2 culture conditions, Y27632-treated cultures ($+Y_T$) showed a slightly higher, ~5-fold increase in GAG production compared to untreated cultures ($+Y_T$) (Fig. 5.2F&G, green bars). These results suggest that the stimulatory effect of the ROCK signalling inhibition during phase II was enhanced slightly by temporal FGF signalling compared to continuous treatment. The results also confirm the importance of ROCK signalling inhibition during phase II, since there were no differences in temporal versus continuous FGF2 treatment in the absence of ROCK inhibition.

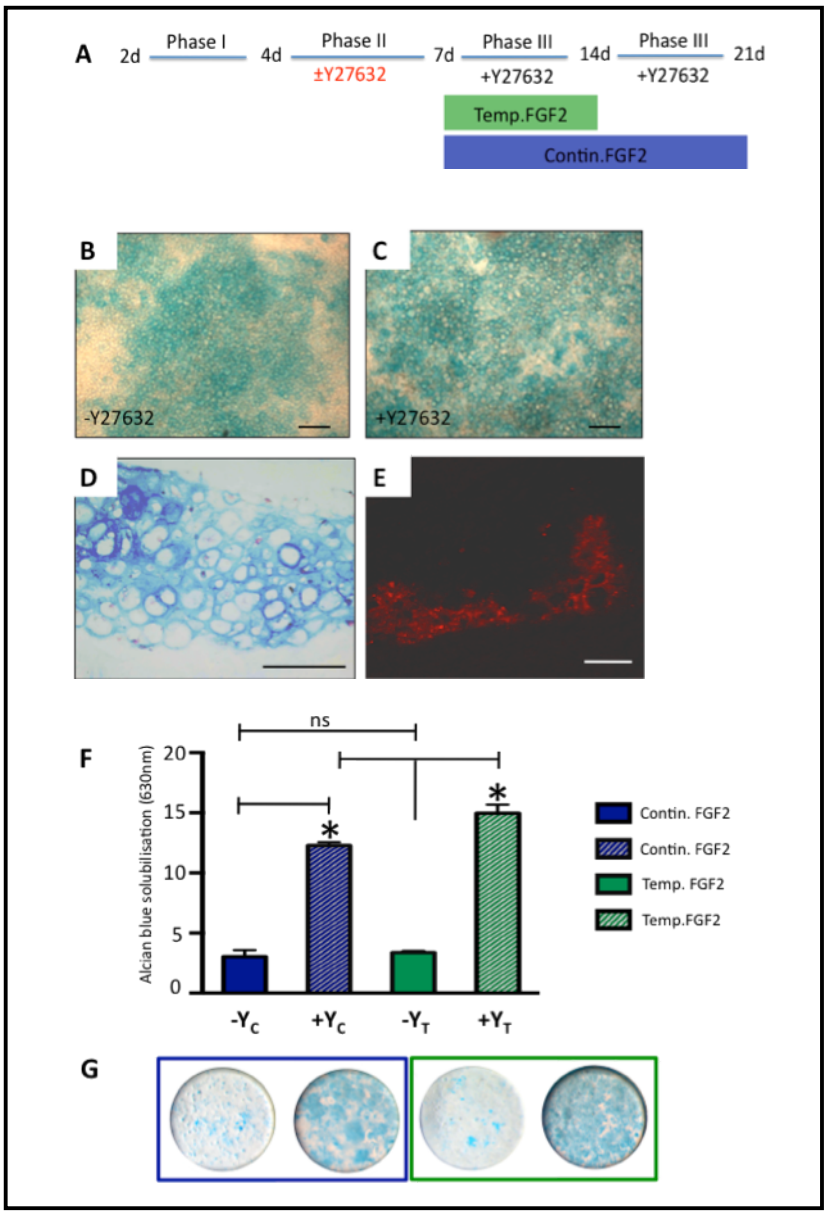


Figure 5.2: The effect of ROCK inhibition and temporal FGF2 on chondrogenic differentiation.

A) A differentiation scheme showing the timing of factors additional to the culture media in phases II and III. In phase II, cells were cultured in the presence of absence of Y27632. In phase III, cells were cultured in presence of FGF2, BMP4, and Y27632. FGF2 was removed from the medium either at 14d (temporal, labelled Temp.FGF2) or at 21d (continuous, labelled contin. FGF2) while cells were cultured in continuous presence of BMP4 for 21d. B&C) Alcian blue stained monolayers showing larger and rounder hypertrophic chondrocyte morphology in Y27632-treated compared to untreated cultures. D) A cross-section of a monolayer stained with H & E and Alcian blue showing abundant extracellular matrix and large round chondrocyte morphology. E) Collagen type X immunofluorescence staining of a monolayer. F) Alcian blue stained wells were solubilised for quantification. G) Alcian blue staining of monolayers from temporal and continuous FGF2 cultures treated in the presence and absence of Y27632. Data indicate mean ±SD (triplicates) of one experiment (paired student t-test; *$p<0.05$). (±Y_C: indicate continuous FGF2),(±Y_T: indicate temporal FGF2).

Subsequently, molecular analysis demonstrated under continuous FGF2 culture conditions that Y27632 treatment ($+Y_C$) did not have any effects on Sox9 expression; however, it led to a 10-fold increase in both Col II and Col X expression compared to untreated cultures ($-Y_C$) (Fig. 5.3, blue bars). Interestingly, in temporal FGF2 culture conditions, Y27632 treatment ($+Y_T$) upregulated Sox9 expression by 1.6-fold with a 10.5-fold and a 30-fold increase in the expression of Col II and Col X, respectively compared to untreated cultures ($-Y_T$) (Fig. 5.3, green bars). These results suggest that ROCK signalling inhibition together with temporal FGF2 signalling enhanced both chondrogenic precursors and mature chondrocyte differentiation.

Interestingly, in the absence of Y27632, temporal FGF2 cultures (Fig. 5.3; $-Y_T$, green bars) did not have an effect on Sox9 expression, however, there was an upregulation of Col II and Col X expression in comparison to continuous FGF2 cultures (Fig. 5.3; $-Y_C$, blue bars). Therefore, the results confirm the Alcian blue staining (Fig. 5.2G) and the importance of ROCK inhibition during phase II in the stimulation of chondrogenic differentiation, in particular, in promoting chondrogenic or maybe chondro-osteoprecursor differentiation, since there were no differences in temporal vs continuous FGF2 treatment in the absence of ROCK inhibition (i.e. $-Y_T$ vs $-Y_C$).

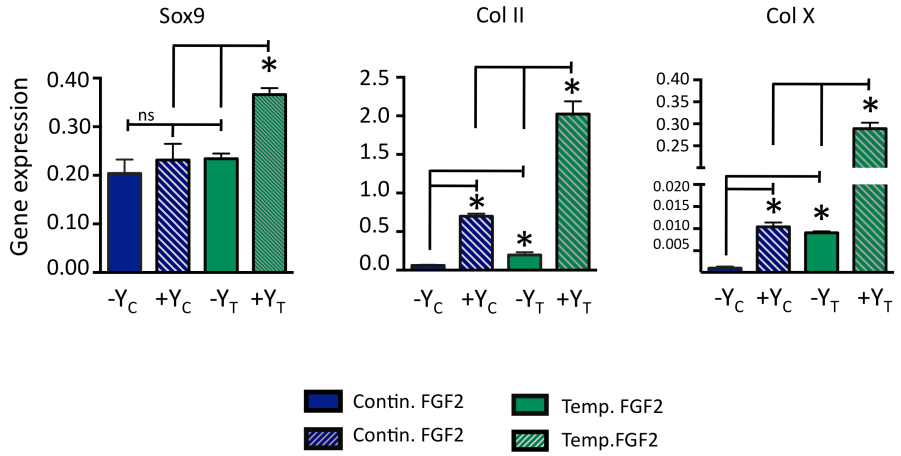


Figure 5.3: The effect of ROCK inhibition and temporal FGF2 on chondrogenic gene expression.
RNA was extracted from continuous and temporal FGF2 cultures from Y27632 treated (+Y) and untreated (-Y) cultures for qPCR analysis of chondrogenic marker gene expression, Sox9, Col II and Col X. Data indicate mean ±SD from one representative experiment from 2 independent experiments (paired student t-test; *p< 0.05) ($\pm Y_C$: continuous FGF2), ($\pm Y_T$: temporal FGF2).

5.3.2 The effect of ROCK inhibition and GDF5 on chondrogenic differentiation and maturation.

In the previous section it was demonstrated that Y27632 treatment in temporal FGF2 culture conditions enhanced chondrocyte maturation towards hypertrophy. Hatakeyama et al. (2004) and Craft et al. (2013) reported that BMP4 and GDF5 controlled the hypertrophic and non-hypertrophic differentiation of chondrocytes in E11.5 mouse micromass cultures and ESC cultures, respectively. Therefore, in this project, it was investigated whether manipulation of BMP4 and GDF5 would influence chondrogenic differentiation (i.e. hypertrophic vs non-hypertrophic chondrocytes) and whether inhibition of ROCK would have an effect on either condition. Therefore, cells were cultured in the presence of either BMP4 or GDF5 in phase III. FGF2 was removed after 1 week of culture and an analysis of cultures was performed after another one week in culture (Fig. 5.4A).

Cultures treated with GDF5 reached confluence in 4 days and chondrocyte nodule formation developed more rapidly in GDF5 cultures compared to BMP4 cultures (data not shown). At the morphological level, GDF5 cultures showed distinctive tightly packed, chondrocyte nodules (Fig. 5.4B) and the Y27632-treated cultures resulted in very large 3-dimensional nodules (Fig. 5.4C) that showed increased matrix deposition/GAG synthesis, as measured by quantification of Alcian blue staining, in comparison to the untreated cultures ($\pm Y_G$)(Fig. 5.4D&E, pink bars). Therefore, the results suggest that GDF5 signalling stimulated chondrogenic differentiation and aggregation and inhibition of ROCK signalling enhanced it.

In comparison to BMP4 cultures, there were clear differences in morphology and distribution of chondrocyte nodules between BMP4 (Fig. 5.2B&C) and GDF5 cultures (Fig. 5.4B&C). The Alcian blue quantification results showed that in the absence of Y27632 in phase II, GDF5 cultures ($-Y_G$) showed a significant increase in GAG production compared to BMP4 cultures ($-Y_B$) (Fig. 5.4D&E), suggesting that GDF5 signalling enriched the number of chondrocyte nodules more than BMP4 signalling.

While upon the addition of Y27632 in phase II, GDF5 cultures ($+Y_G$) showed no significant differences from BMP4 cultures ($+Y_B$) (Fig. 5.4D&E), although the distribution of nodules were markedly different.

Taken together, ROCK inhibition during the mesoderm enrichment phase enhanced the chondrogenic effects of both BMP4 and GDF5. However, in contrast to BMP4, GDF5 signalling stimulated the number of chondrocyte nodules even without ROCK inhibition in phase II. Despite the dramatic differences in chondrocyte nodule morphology and distribution in GDF5 versus BMP4 cultures, total matrix accumulation was similar, suggesting that there were additional cellular or molecular differences between the two growth factors.

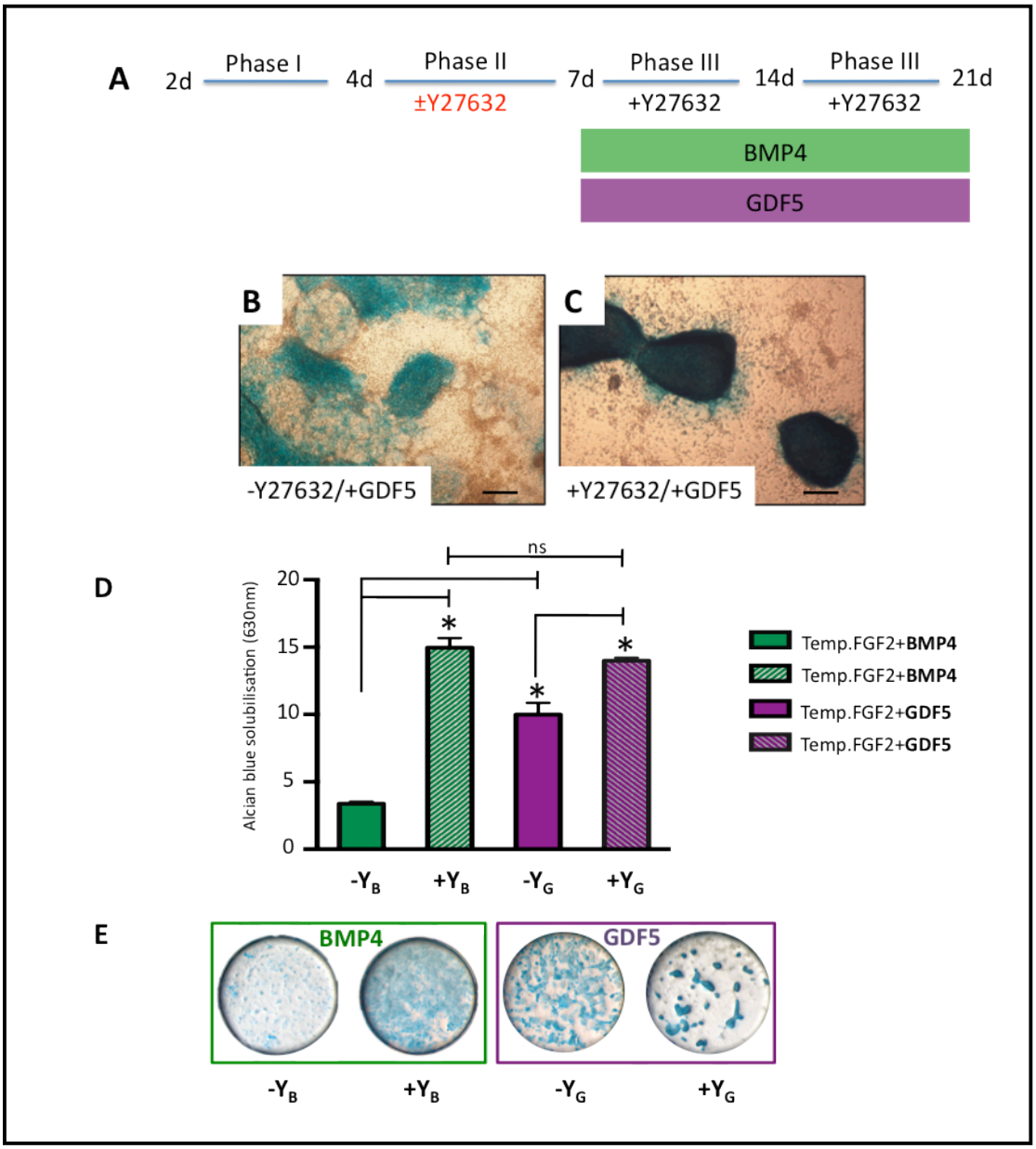


Figure 5.4: The differences between BMP4 and GDF5 signalling in ROCK – dependent chondrogenic differentiation.

A) A differentiation scheme showing the timing of the addition/removal of factors in the culture media in phases II and III. In phase II, cells were cultured in FGF2 and Y27632. Then in phase III, cells were cultured in the presence of FGF2 and Y27632 with either BMP4 or GDF5 for 2 weeks, whilst at 14d, FGF2 was removed. B&C). B&C) Alcian blue stained monolayers from GDF5 cultures cultured in the absence (B) or presence (C) of Y27632 during phase II. D) Alcian blue stained wells were solubilised for quantification. E) Images of Alcian blue stained monolayers from BMP4 ±Y27632 (green box) and GDF5(pink box) cultures. Data indicate mean ±SD (triplicates) from one experiment (triplicates)(paired student t-test;*p<0.05) (±Y_B: BMP4), (±Y_G**:** GDF5).

To further analyse the effect of both Y27632 and GDF5 treatment on chondrogenic differentiation, preliminary molecular analysis of chondrogenic marker gene expression was undertaken after a short culture period of 1 wk and was compared to BMP4-treated cultures. The results revealed that in Y27632-treated cultures, BMP4 ($+Y_B$) stimulated Acan and Col X expression as shown earlier (Chapter 4, Fig. 4.6B) and additionally, stimulated the articular cartilage marker Prg4/Lubricin compared to Y27632 untreated cultures ($-Y_B$) (Fig. 5.5A). However, in the presence of GDF5 ($+Y_G$), there was a downregulation in Acan and Col X expression but an upregulation of Prg4/Lubricin compared to untreated cultures ($-Y_G$) (Fig. 5.5B). These preliminary results suggest that GDF5 and ROCK signalling might interact to drive chondrogenic differentiation towards a more "articular-like" cartilage phenotype.

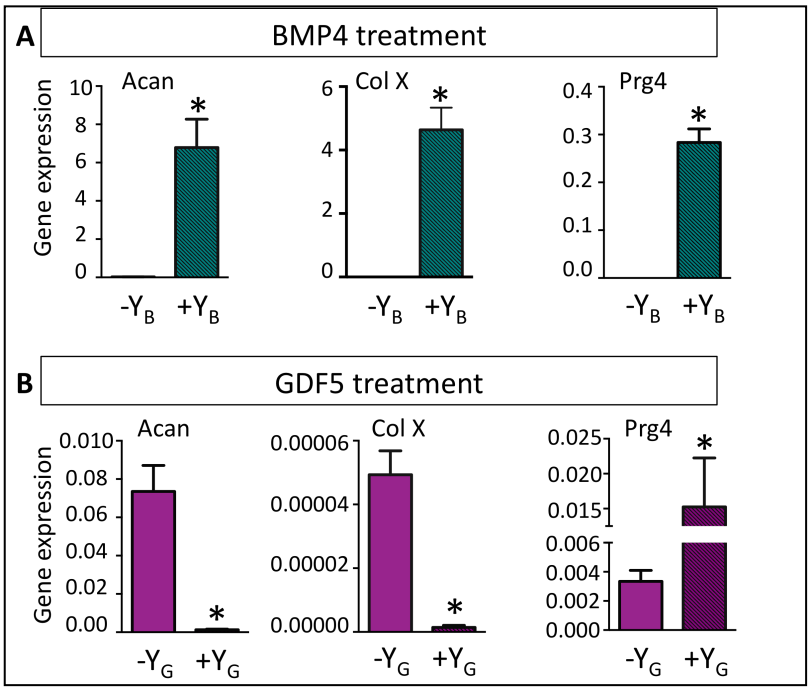


Figure 5.5: The effects of BMP4 and GDF5 on chondrogenic gene expression.

RNA was extracted at 14d from the (A) BMP4 and (B) GDF5 cultures that were Y27632 treated (+Y) or untreated (-Y) in phase II. qPCR analysis was performed for the chondrogenic marker genes Acan, Col X (hypertrophic chondrocyte marker) and Prg4 (articular chondrocyte marker). Data indicate mean ±SD from one representative experiment from one experiment (paired student t-test; *p< 0.05).

5.4 Discussion

In this chapter, two aspects of chondrogenic differentiation have been examined. First, the finding in chapter 4 that the effect of phase–specific inhibition of ROCK signalling could enhance chondrogenic maturation and hypertrophy, as evidenced by Col X, prompted further optimisation and characterisation of these populations.

The chondrogenic differentiation media used so far contained FGF2 and BMP4, based on developmental studies for limb and endochondral growth plate development which showed distinct and sometimes antagonistic effects of FGF signalling on chondrocyte lineage commitment, proliferation and differentiation (Yoon et al. 2006; Sahni et al. 1999). Several studies have reported that FGF2 stimulates proliferation while inhibiting hypertrophic differentiation in cultures of rat rib growth plate chondrocytes (Wroblewski & Edwall-Arvidsson 1995), and in micromass of rabbit rib cells (Kato & Iwamoto 1990). Therefore, it was reasoned that continuous exposure of the ESC-derived chondrocytes to FGF2, despite the presence of BMP4, might not provide the optimal conditions for cartilage differentiation in the ESC/EB culture system used in this project. Hence, the hypothesis was that the effects of ROCK inhibition might be better documented under optimal differentiation.

The time period of FGF signalling was shown to be critical and the stimulatory effect of ROCK inhibition on chondrogenesis was enhanced by the withdrawal of FGF signalling at the latter stages of chondrocyte differentiation. Temporal FGF2 signalling enhanced the chondrogenic maturation effect of ROCK signalling inhibition, as evidenced by matrix deposition and Col II, Col X gene expression. However, the importance of ROCK signalling inhibition during phase II was shown to be its role in promotion of chondrogenic or perhaps chondro-osteoprogenitor differentiation, regardless of the duration of exposure to FGF2 signalling. Other reports have shown that ROCK inhibition stimulates Sox9 expression (as discussed in chapter 4); however, there have been no reports on the effect of temporal FGF and phase–specific ROCK signalling inhibition, which is unique to this project.

The preliminary results from culturing cells in GDF5 demonstrate that the culture conditions could be manipulated by BMP4 and GDF5 signalling to recapitulate hypertrophic and non-hypertrophic chondrocyte differentiation, respectively. The improved culture conditions (i.e. temporal FGF2 exposure) were used for assessing the effects of GDF5 as a potential inducer of an articular cartilage phenotype. ROCK signalling inhibition combined with GDF5 signalling stimulated a chondrogenic population with distinctive morphology, enhanced matrix deposition, and articular–like gene expression, demonstrated by the upregulation of Prg4/Lubricin expression and the downregulation of Col X expression compared to ROCK signalling inhibition when combined with BMP4 signalling. Gene expression was performed on a 1-week culture, and further experiments looking at longer-term cultures will determine whether GDF cultures can maintain a stable articular/non-hypertrophic phenotype.

These outcomes were consistent with the findings reported previously using different culture models (Hatakeyama et al. 2004; Craft et al. 2013). Hatakeyama et al. (2004), which showed, in micromass cultures of E11.5 cells, a differences in chondrocyte morphology between GDF5 and BMP4-stimulated chondrocyte nodules. In addition, in a mESC, serum free culture, Craft et al. (2013) demonstrated similar differences in chondrocyte morphology in cells cultured in BMP4 and GDF5, whereby GDF5 upregulated Prg4 expression and low levels of Col X expression were found in comparison to BMP4 cultures.

It seems that inhibition of ROCK signalling, in particular during the mesoderm enrichment phase (+/-), induced hypertrophic chondrocyte differentiation in the presence of BMP4 combined specifically with temporal FGF2 signalling. In addition, independently of FGF2 signalling, ROCKsignalling inhibition seems to enrich for chondrogenic and/or chondro-osteoprogenitor cells. Finally, ROCK signalling inhibition together with GDF5, but not with BMP4 signalling, might be inducing a differentiation programme that resembles a non-hypertrophic (articular-like) chondrocyte differentiation.

Consequently, access to enriched populations of hypertrophic and non-hypertrophic chondrocytes developed by manipulation of FGF2, BMP4 or GDF5 together with stage-specific ROCK inhibition will provide insights into molecular events regulating both hypertrophic and non-hypertrophic differentiation under the influence of the ROCK pathway and their potential future use in tissue replacement therapy for the treatment of degenerative diseases such as osteoarthritis.

Chapter 6 Effect of ROCK inhibition on osteogenic differentiation of ESC-derived mesoderm

6.1 Part A

6.2 Introduction

During embryonic development of endochondral bone, a cartilage template is formed which is then replace by bone (Hall & Miyake 2000; Olsen et al. 2000; Goldring et al. 2006; Long & Ornitz 2013b). Bone as a tissue is composed of 3 cell types; osteoblasts (bone-forming cells), osteocytes (terminally differentiated osteoblasts engulfed in mineralized bone matrix), and osteoclasts (bone-resorbing cells). Osteoblasts secrete extracellular matrix, which consists of mineral (hydroxyapatite), collagen (predominantly collagen type I (herein Col I)), water, noncollagenous proteins, and lipids in decreasing proportion (Boskey&Robey 2013). Bone formation (osteogenesis) is a multistep process that is controlled by a cascade of molecular events (Karsenty et al. 2009).

Small GTPAse signalling has been shown to influence osteogenesis, in terms of: lineage commitment (McBeath et al. 2004), osteogenesis marker gene expression, differentiation and maturation (Ohnaka et al. 2001; McBeath et al. 2004; Harmey et al. 2004; Yoshikawa et al. 2009; Khatiwala et al. 2009; Prowse et al. 2013). Specifically, the role of Rho/ROCK signalling in osteogenesis appears to be complex. Several reports have suggested a positive role for Rho/ROCK signalling in osteogenesis commitment and differentiation (McBeath et al. 2004; Meyers et al. 2005; Khatiwala et al. 2009; Arnsdorf et al. 2009). However, others have shown the contrary, specifically using the Y27632 chemical inhibitor (Ohnaka et al. 2001; Harmey et al. 2004; Kanazawa et al. 2009; Yoshikawa et al. 2009; Prowse et al. 2013).

The effect of ROCK signalling on osteogenesis had been investigated in our lab (Harmey et al. 2004). In primary mouse calvarial osteoblast cultures, our laboratory has shown that the inhibition of ROCK signalling positively influenced the differentiation and maturation of primary osteoblast cells and while activation of Rho by the Pasteurella Multocida Toxin (PMT), led to a decrease in osteoblast differentiation, suggesting a role of Rho/ROCK in osteoblast differentiation. Interestingly, the Harmey et al. (2004) study pointed out that the effect of ROCK inhibition was dependent on the differentiation stage of the cells and of nodule formation, suggesting that ROCK may be targeting the differentiation of osteoblast precursors rather than matrix deposition and

mineralisation. Collectively, these studies highlighted the complexity of ROCK signalling, which may be further explained by several possibilities, such as species differences, culture conditions (e.g. 2D versus 3D cultures), cell origin and the stage of cell development during the differentiation process. Thus, the exact mechanism of ROCK signalling in regulating osteogenic development is not yet clear and unravelling the complexity of this signalling pathway in osteogenic differentiation has been an interest in the field of musculoskeletal diseases such as osteoarthritis. Therefore, establishing an ESC/EB, step-wise, mESC mesoderm differentiation model system (discussed in chapter 3) allows for a controlled and defined method for dissecting and further understanding of the molecular events occurring during development of bone, in particular, specification and differentiation. Thus, this chapter will investigate the effect of ROCK inhibition, specifically at two developmental stages: the mesoderm enrichment phase (phase II) and the differentiation phase (phase III).

6.3 Methods

The 3-phase ESC/EB differentiation model system as discussed in chapter 3 was also applied here. The same protocol was followed as was used for the chondrogenic differentiation studies described in chapter 4, except that in phase III, cells were re-plated as single cells in a monolayer culture in osteogenic media (OM) containing 50ug/ml Ascorbic Acid (AA), 10mM β-glycerophosphate (β-GP), 10^{-7}M Dexamethasone, 100ng/ml BMP4 and 10ng/ml FGF2. To test ROCK inhibition, 10mM Y27632 was added to the cultures, as will be shown in each section. Cultures were fixed after 2, 3, or 4 weeks and analysis was undertaken by monitoring the morphological changes of cells in culture, as well as quantification of ALP positive nodule areas, and assessment of mineralisation by von Kossa staining. Molecular analysis of osteogenic marker gene expression (Runx2, Osx, ALP, BSP and OC) was also performed by qPCR.

6.4 Results

6.4.1 Osteogenic differentiation of ESC-derived mesoderm

In chapter 3, molecular analysis showed that both Runx2 and Osx were upregulated after culturing cells in FGF2 for 3 days during the mesoderm enrichment phase (phase II) (chapter 3, Fig. 3.10). Therefore, the next step was to examine whether cells generated at phase II had the functional potential to differentiate towards the osteogenic lineage, when cultured in osteogenic media in the monolayer differentiation phase (phase III) (Fig. 6.1A).

Firstly, morphological analysis under basal osteogenic media conditions indicated that cells started to cluster and form 3-D nodules by 21d and were positively stained with ALP (Fig. 6.1B-E), with a distinctive cuboidal morphology (Fig. 6.1D,E). In addition, the cells expressed collagen type I protein when assessed by immunofluorescence staining (Fig. 6.1F,G). Interestingly, although collagen type 1 was expressed, there were no signs of mineralisation at this period of differentiation, i.e. after 2 wks of differentiation. These results implied that ESC-derived mesoderm differentiated towards the osteogenic lineage, but within this culture period, did not attain a functional stage in order to mineralise the matrix.

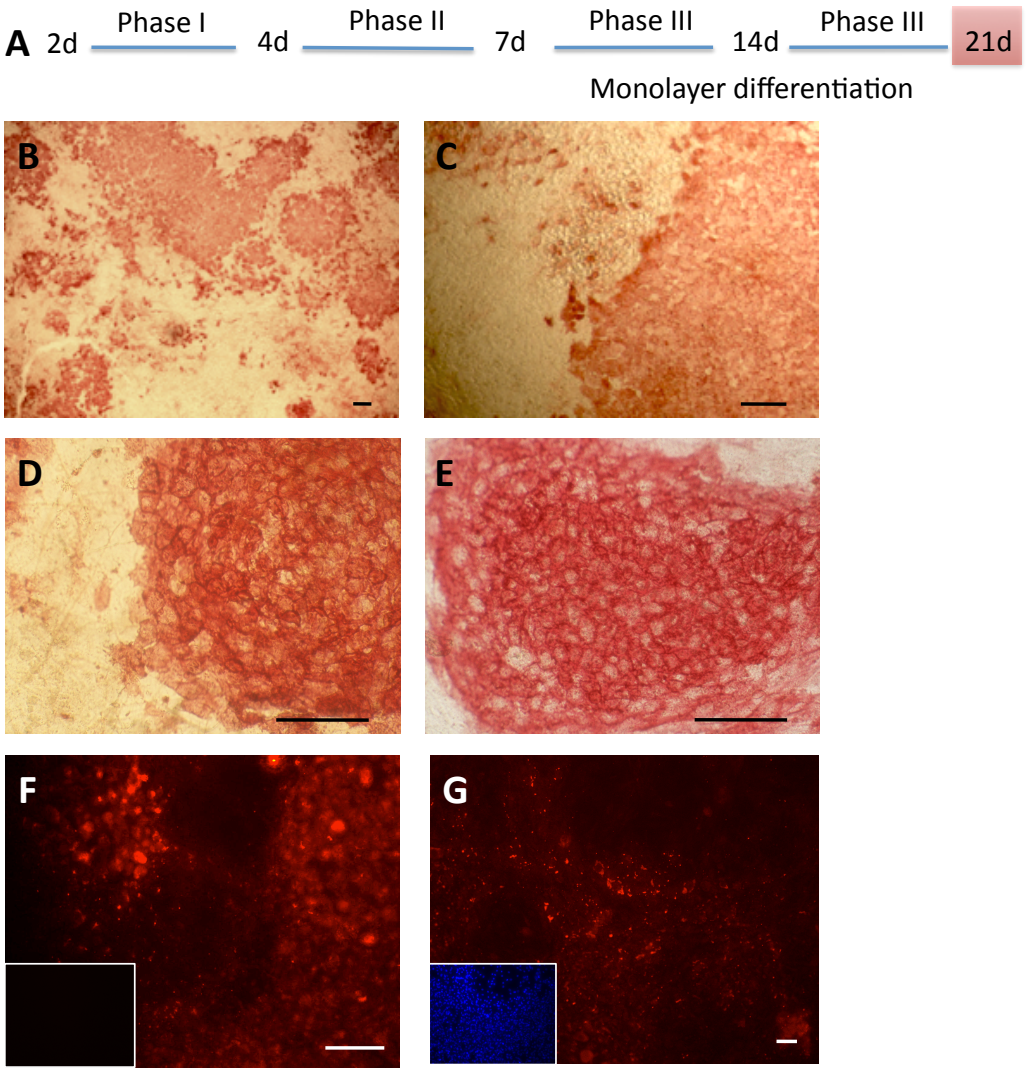


Figure 6.1: Osteogenic differentiation of ESC-derived mesoderm in the monolayer differentiation phase (phase III).

A) A differentiation scheme showing the timing of osteogenic monolayer differentiation. In phase III, cells were cultured in osteogenic media (FGF2 (10ng/ml), BMP4 (100ng/ml), Dex. (10^{-7}M), β-GP (10mM). Monolayer cultures were fixed after 2 weeks for analysis. B,C) ALP staining of osteoblast nodule areas. D,E) Images of 3-D osteoblast nodules showing typical cuboidal morphology of osteoblast cells. F,G) Collagen type I immunofluorescence staining of osteoblast nodules, insert (F; negative control) insert (G; cells stained with Dapi). All images were from representative wells from 10 experiments. Scale bars: 100 µm.

Molecular analysis of cultures at (21d) of differentiation (Fig. 6.2A) showed a significant upregulation of Runx2, Osx, and ALP expression compared to cultures prior to the beginning of monolayer differentiation i.e. at 7d (Fig. 6.2B). Collectively, these results suggested that ESC-derived mesoderm was able to differentiate efficiently towards the osteogenic lineage.

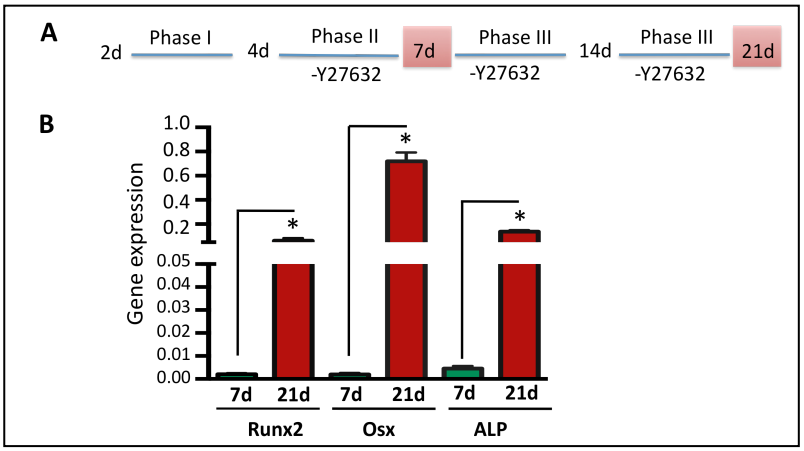


Figure 6.2: Expression of osteogenic markers after 2 weeks in the monolayer differentiation phase (phase III).

A) A differentiation scheme showing cells cultured in the absence of Y27632 in phases II and III, then cultured in osteogenic media in phase III. RNA was extracted at two time points (pink box) during the differentiation period: 7d and 21d. B) Quantitative PCR analysis of osteogenic marker gene expression (Runx2, Osx and ALP). Data indicate mean ±SD (triplicate samples), from one representative experiment of 3 independent experiments. Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (paired student's t-test; * $p < 0.05$, 7d vs 21d).

6.4.2 Effect of ROCK inhibition in the mesoderm enrichment phase (phase II) on osteogenic differentiation

In chapter 3, Y27632 treatment in the mesoderm enrichment phase (phase II) illustrated a differential effect on expression of osteogenic transcription factors (Runx2 and Osx) compared to the untreated cultures. Y27632 treatment did not affect Runx2 expression, however, it downregulated expression of Osx (Fig. 3.13A). To further investigate the functional potential of the Y27632-treated cells, cells were re-plated in phase III in osteogenic media and then cultured for a further 2 weeks (Fig. 6.3A). Quantification of ALP-positive nodule areas indicated that treatment with Y27632 during phase II alone significantly decreased the ALP positive areas compared to Y27632 untreated cultures (Fig. 6.3B&C).

Subsequently, at the molecular level, qPCR analysis indicated that Y27632 treatment downregulated expression of Osx, ALP, BSP while it did not have an effect on the expression of Runx2 and OC (Fig. 6.3D). These results advocate that inhibition of ROCK signalling at the mesoderm enrichment phase (4-7d) inhibited osteogenic differentiation of ESC derived mesoderm in the differentiation phase (7-21d).

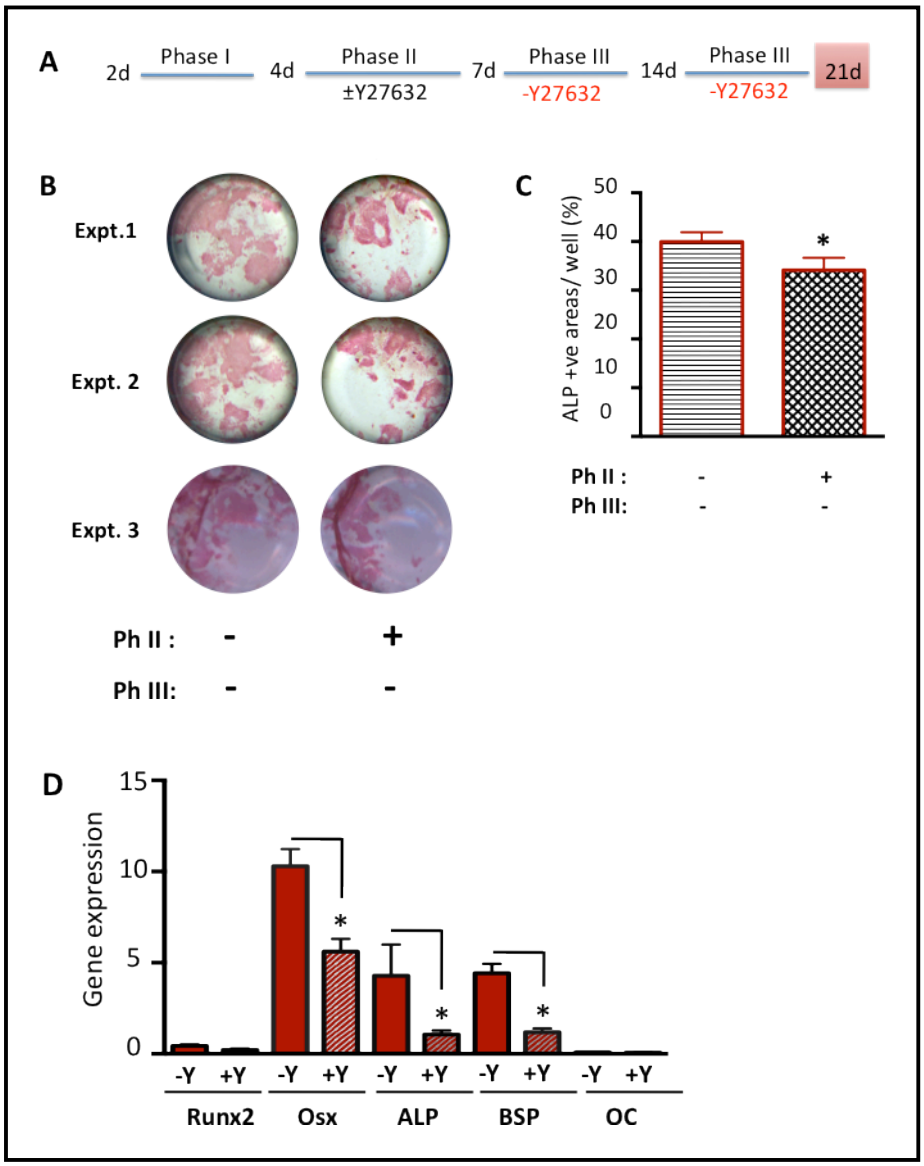


Figure 6.3: Effect of ROCK inhibition in phase II on osteogenic differentiation of ESC-derived mesoderm.

A) A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. In phase III, cells were cultured in osteogenic media for 2 weeks. B) Representative images of ALP stained monolayers from 3 independent experiments. C) Quantification of ALP positive nodule areas. D) Quantitative PCR analysis of osteoblast-specific markers, Runx2, Osx, ALP, BSP and OC at 21d of differentiation. Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (paired student's t-test; * $p < 0.05$).

6.4.3 Effect of ROCK inhibition in both phases II and III on osteogenic differentiation

Following on from the results of the previous section, it was then investigated whether prolonged ROCK inhibition would influence osteogenic differentiation. In phase II, cells were cultured in the presence and absence of Y27632. Then in phase III, cells were cultured in osteogenic media, however, this time cells would be cultured in the presence of Y27632 for 2 weeks (Fig. 6.4A).

Quantification of ALP staining demonstrated a 1.4 fold increase in ALP positive nodule areas when cells treated with continuous Y27632 in comparison to the control (Fig. 6.4B,C). In parallel, continuous Y27632 treatment showed an upregulation of mature osteogenic markers (BSP), while it showed no effect on (Runx2, Osx, ALP and OC) compared to the control cultures (Fig.4D). These results indicated that the continuous inhibition of ROCK signalling in both phases (4-21d) had stimulated osteogenic differentiation of ESC-derived mesoderm and possibly had directed the differentiation towards maturation.

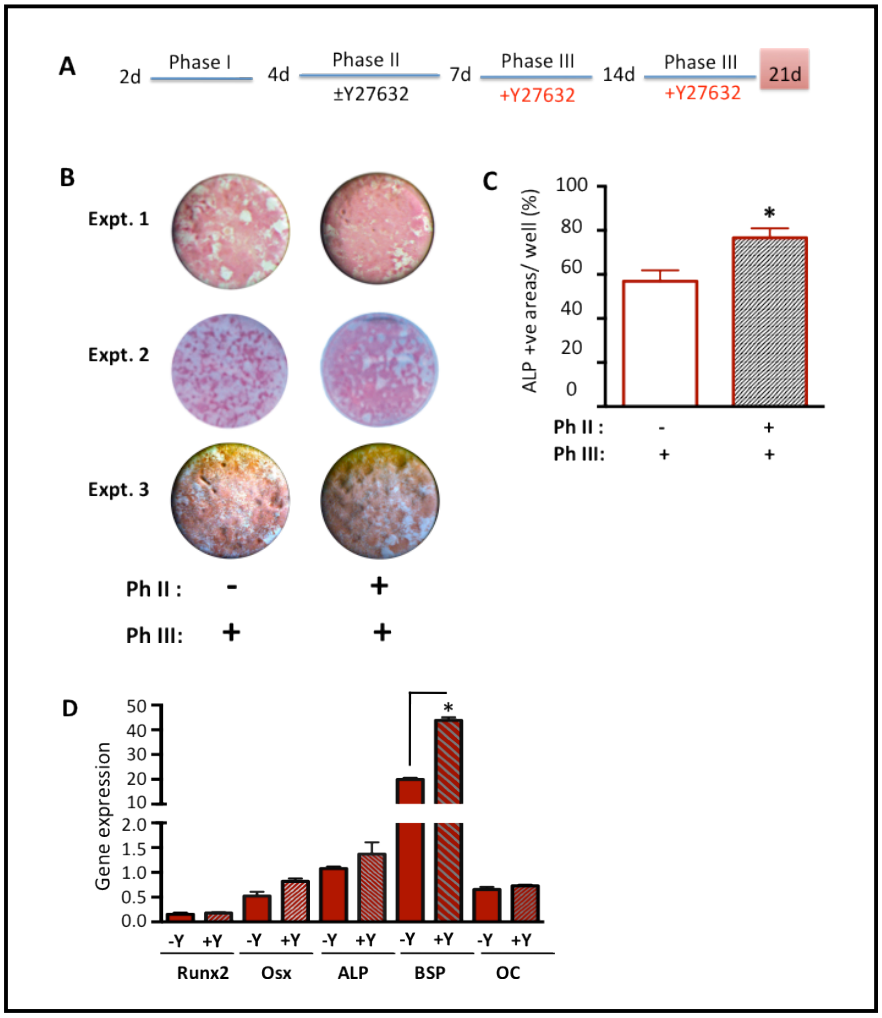


Figure 6.4: Effect of continuous ROCK inhibition in both phase II and III on osteogenic differentiation of ESC- derived mesoderm.

A) A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. In phase III, cells were cultured in osteogenic media for 2 weeks. B) Representative images of ALP-stained monolayers from 3 independent experiments. C) Quantification of ALP-positive nodule areas. D) Quantitative PCR analysis of osteoblast-specific markers (Runx2, Osx, ALP, BSP and OC) at 21d of differentiation. Data indicate mean ±SD (triplicate samples), from one representative experiment of 3 independent experiments. Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (paired student's t-test; * $p < 0.05$).

Summarising all four treatment groups together (Fig. 6.5A), ALP staining results showed a 2-fold increase in ALP positive areas in continuous Y27632-treated cultures compared to untreated cultures (-/- vs +/+), followed by a 1.4-fold increase in cultures exposed to a temporal Y27632 treatment in phase III only (7-21d) compared to untreated cultures (-/- vs -/+). Interestingly, temporal Y27632 treatment in phase II only caused a slight but significant decrease in ALP positive areas compared to untreated cultures (-/- vs +/-) (Fig. 6.5B&C).

These results confirmed that temporal inhibition of ROCK signalling in phase III (-/+) is the key in inducing osteogenesis, and continuous ROCK signalling inhibition (+/+) enhanced the differentiation and maturation processes. These results suggest a developmental phase-specific effect of ROCK signalling inhibition on osteogenic differentiation.

Moreover, BSP expression results proved that continuous Y27632 treatment (+/+) and temporal Y27632 treatment in phase III only (-/+) upregulated BSP expression by 6-fold and 5-fold respectively, compared to untreated cultures. By contrast, Y27632 treatment in phase II only (+/-) showed a significant 3-fold reduction in BSP expression compared to untreated cultures (-/-) (Fig. 6.5D).

Taken together, and in comparison to the chondrogenic effect of Y27632 treatment seen in chapter 4 (Fig. 4.7), it seems that ROCK inhibition in phase II (+/-) differentially affects chondrogenic and osteogenic differentiation, and continuous ROCK inhibition (+/+) directs towards chondrocyte hypertrophy and further osteoblast maturation.

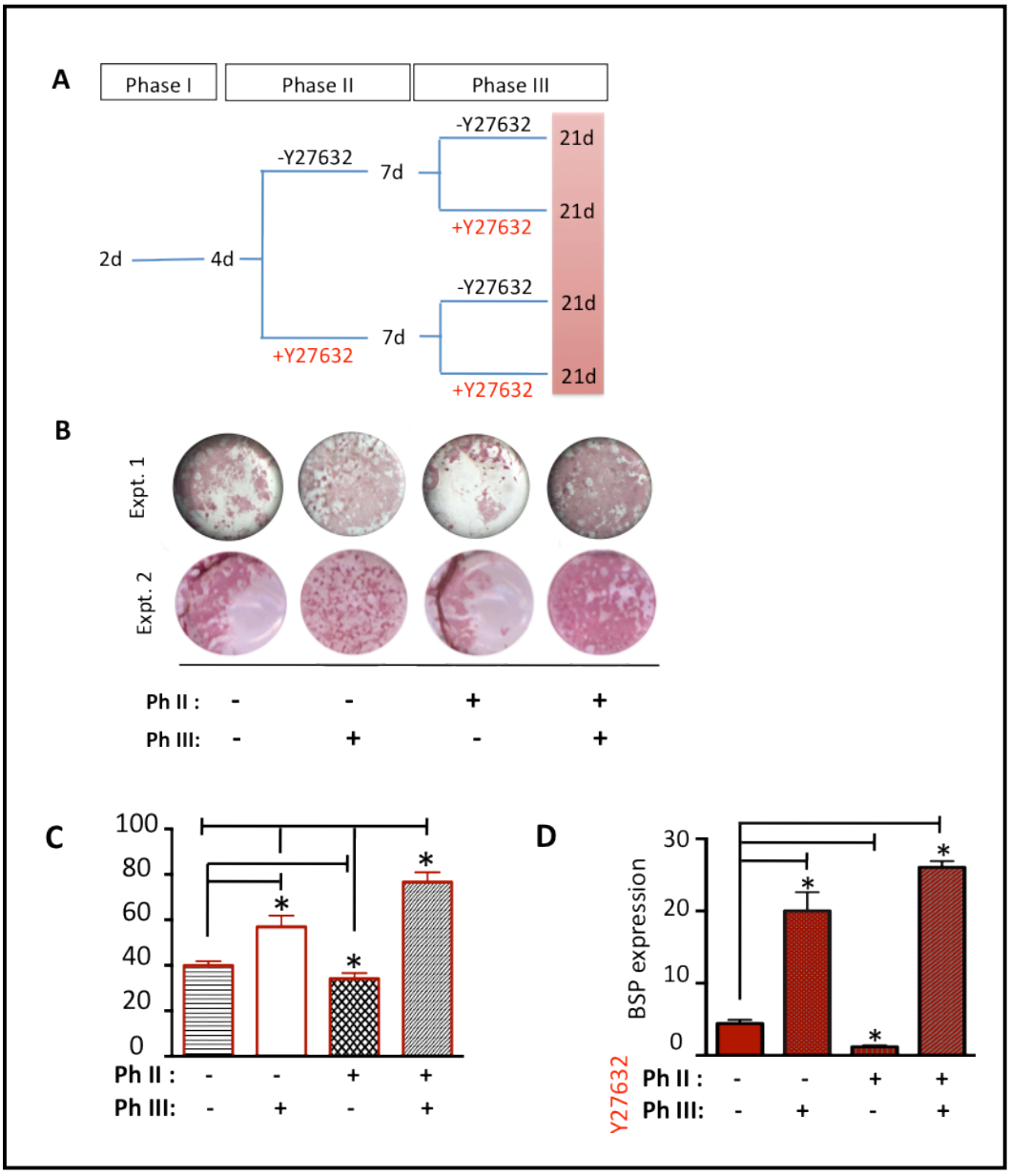


Figure 6.5: Effect of stage-specific ROCK inhibition in phases II and III on osteogenic differentiation of ESC-derived mesoderm.

A) A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. In phase III, cells were cultured in osteogenic media for 2 weeks. B) Representative images of ALP-stained monolayers from 2 independent experiments. C) Quantification of ALP-positive nodule areas. D) Quantitative PCR analysis of osteoblast specific markers, BSP at 21d of differentiation. Data indicate mean ± SD (triplicates) from a representative experiment of 3 independent experiments (one-way ANOVA; * $p < 0.05$).

6.5 Discussion

In the first part of this chapter (partA), it was demonstrated that the serum-free, 3-phase ESC/EB model system effectively directed ESCs towards osteoblast differentiation. As mentioned in the introduction, the effect of ROCK signalling on osteogenesis is complex, and according to cell types derived from different species, the differentiation stage of the cells, and culture conditions, all of which could influence the role of Rho-ROCK signalling. The use of the ESC/EB differentiation protocol (chapter 3) enabled the role of ROCK inhibition on osteoblast differentiation to be addressed in a defined and controlled manner, by analysing the effects of ROCK inhibition at two developmental stages: mesoderm enrichment (phase II) and differentiation phases (phase III).

Herein, it was proved that temporal ROCK inhibition indeed has a developmental phase-specific effect on osteogenic differentiation. When ROCK was inhibited during the mesoderm enrichment phase only (4-7d), osteogenesis was not induced. However, when ROCK was inhibited, either during the differentiation phase only (-/+) or continuously in both phases (+/+), osteogenesis was induced. These findings will be discussed further in the following sections.

Upon temporal/short-term inhibition of ROCK signalling in phase II only (+/-), there was a reduction in the percentage of ALP positive nodule areas and this was accompanied by a downregulation in the expression of Osx, ALP and BSP. Similar results were shown in other reports (McBeath et al. 2004; Khatiala et al.2009; Arnsdorf et al. 2009). The McBeath et al. (2004) study demonstrated that RhoA/ROCK signalling is required for osteogenic differentiation of hMSC and that ROCK is downstream of both cell shape and soluble factors. Expressing constitutively active RhoA in un-spread cells led to an osteogenic fate while spread cells differentiated to an adipogenic fate, suggesting an effect of cell shape in the osteogenic effect of RhoA. However, constitutively active Rho effector, ROCK, induced osteogenesis in both round and spread cells, independently of cell shape and Y27632 showed the opposite effect.

Therefore, supporting the importance of ROCK signalling in the osteogenic differentiation of hMSCs. Therefore, it could be speculated that the inhibitory effect of ROCK on osteogenesis seen in our cultures may suggest that the cells generated from exposing phase II cultures to ROCK inhibition (+/-) could have been in a MSC-like stage and therefore had responded negatively to ROCK inhibition. Alternatively, as phase II cultures are non-adherent aggregates, the consequences of cell shape cannot be ruled out.

In contrast to the short-term effects of ROCK inhibition in phase II (+/-) on long-term osteoblast differentiation, it was demonstrated that continuous ROCK inhibition, in both phases (+/+), stimulated osteogenesis which was manifested by the increase in the ALP-positive nodule area and tended towards an upregulation of bone specific markers (Runx2, ALP) while significant increase in BSP expression. This positive ROCK inhibition effect on osteogenesis in phase III was also verified when ROCK was temporally inhibited in phase III only (-/+). These results confirm that ROCK inhibition in phase III is responsible for inducing osteogenesis from ESC-derived mesoderm.

These stimulatory results of ROCK inhibition on osteogenesis correspond with other studies where ROCK inhibition stimulated osteogenesis in pre-osteoblast cultures which might represent more committed cells (Harmey et al. 2004; Yoshikawa et al. 2009; Prowse et al. 2013). Harmey et al. (2004) demonstrated that inhibition of Rho (using C3 transferase) and ROCK (using Y27632 or HA-1077) led to stimulation of osteoblast differentiation and mineralisation, as shown by an increase in bone nodule formation and up regulation of ALP and OC gene expression. Further, this study pointed out that the effect of ROCK inhibition was dependent on the differentiation stage of the cells, i.e. the stage of nodule formation and treatment with ROCK inhibitors may be targeting the differentiation of osteoblast precursors rather than matrix deposition and mineralisation as analysed by ALP quantification.

Similarly, Yoshikawa et al. (2009) proved that ROCK inhibition using Y27632 stimulated osteogenesis both *in vivo* and *in vitro*. Initially, it was demonstrated that Y27632 treatment increased osteogenesis by upregulation of ALP and OC expression and increased nodule formation compared to the untreated cultures.

Further, the osteogenic effect of ROCK inhibition was confirmed by using a constitutively active ROCK mutant and a dominant-negative ROCK construct in mesenchymal ST2 cells. This stimulatory effect was also reproduced in human primary osteoblast cultures using a different ROCK inhibitor, Hydroxyfasudil (Ohnaka et al. 2001). Therefore, the results of our study could suggest that the cells in phase III are at a pre-osteoblast-like stage of development, and hence responded positively to ROCK inhibition.

On the molecular level, the osteogenic effect of ROCK inhibition in the differentiation phase (phase III) was linked to BMP signalling (Harmey et al. 2004; Yoshikawa et al. 2009). This was demonstrated by correlation of the upregulation of ALP and OC expression in Y27632 treated cultures with the upregulation of BMP4 expression using qPCR and Northern blot analyses. In addition, Yoshikawa et al. 2009 demonstrated that ROCK inhibition stimulated ectopic bone formation in the presence of BMP2 in the implanted collagen composite, therefore, suggesting the cooperation between ROCK and BMP signalling in inducting osteogenesis.

Similarly, Hydroxyfasudil upregulated BMP2 expression in MC3T3 cultures (Kanazawa et al. 2009). In addition, other reports have proven the association of ROCK inhibition with the BMP pathway by the use of other classes of ROCK pharmacological inhibitors such as statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (Ohnaka et al. 2001; Kanazawa et al. 2009). Both studies demonstrated that the enhancement of BMP2 and OCN mRNA expression in osteoblasts is induced by hydroxyfasudil using hMSC (Ohnaka et al. 2001) and MC3T3 cultures (Kanazawa et al. 2009). Therefore, the mechanism of osteogenesis in our culture conditions could be related to BMP signalling, especially as the osteogenic media is supplemented with BMP4, yet the exact mechanisms and downstream pathways that might be activated following ROCK inhibition are not yet known and require further investigation.

Moreover, the stage-specific effect of ROCK inhibition on osteogenic differentiation of ESC-derived mesoderm could be explained in part by the expression studies preformed during the mesoderm enrichment phase (phase II: 4t-7d) (chapter3). The findings proved that cells exposed to ROCK inhibitor at phase II (4-7d) expressed markers of a bi-potential chondro-osteoprogenitor

population as well as paraxial and lateral plate mesoderm markers. However, when comparing cultures untreated and treated with ROCK inhibitor, ROCK inhibition at phase II (+/-) proved to differentially affect the differentiation mesoderm and chondro-osteoprogenitor population.

Collectively, this suggests that the generated populations (i.e. exposed to ROCK inhibitor (4-7d)) might respond differently upon subsequent long-term differentiation (7-21d); and indeed there were differences, as discussed in this chapter and in chapter 4 for chondrogenesis. Taken together, it seems that there is a preferential effect of ROCK inhibition in phase II (+/-) towards chondrogenic over osteogenic differentiation, at least according to histochemical analysis. These interesting results, however, require further analysis of both cartilage and bone specific gene markers expression for confirmation.

Findings from long-term/continuous ROCK inhibition in both phases II and III (+/+) enhanced chondrocyte hypertrophy as demonstrated in (chapter 4) and led to advancement in osteoblast differentiation, thereby mimicking a growth plate differentiation process. In addition, BSP expression analysis showed similar pattern under both chondrogenic and osteogenic media conditions (data not shown). BSP is not only expressed during bone differentiation, but it is also expressed in hypertrophic chondrocytes (Chen et al. 1991; Barnes et al. 2002; Gerstenfeld & Shapiro 1996). Thus, it is speculated that the cells generated after a 2-week differentiation phase (phase III: 7-21d) could be at a specific stage that might represent an endochondral ossification process of bone formation where cells might be at the transition stage between late stages of chondrocyte differentiation and hypertrophy and early osteogenic differentiation. The *in vivo* potential of cells will further verify this speculation (chapter 7).

In summary, based on ALP staining quantification and gene expression analysis in this section, it is established that that ROCK inhibition modulates ESC-derived mesoderm differentiation to osteoblast in a phase-specific manner. The use of the ESC/EB differentiation protocol enabled us to address the role of ROCK inhibition in a defined and controlled manner, by analysing the effects of ROCK inhibition at two developmental stages: mesoderm enrichment (phase II) and differentiation phases (phase III). The inhibition of ROCK in the differentiation phase showed to be the key stage for the anabolic effect on

bone, while ROCK inhibition in the mesoderm enrichment phase showed the contrary. Furthermore, the findings here in this chapter and in combination with findings in chapter 4 and 5, strongly suggest that long-term ROCK inhibition manipulation directs the maturation of chondrocytes and osteoblasts, possibly mimicking endochondral ossification. The ability of the osteoblasts to mature further and attain full mineralisation *in vitro* - which is a characteristic of functional bone tissue - will be interesting to investigate further and this will be discussed in the next part of this chapter (part 6.B).

In conclusion, this is the first evidence for the osteogenic differentiation from ESC-derived mesoderm using the novel 3-phase, serum-free ES/EB differentiation system. In addition, appreciating the influence of the developmental stage of cells on the osteogenic effect of ROCK signalling may pave the way for further comprehensive understanding of the molecular events regulating the ROCK pathway and provide future consideration for potential therapeutic targets for metabolic diseases characterised by bone loss such as osteoporosis.

6.6 Part B

6.7 Introduction

During bone formation, in order for bone tissue to become functional, it undergoes a stage of mineralisation to strengthen the collagen composite of bone. This provides mechanical resistance to bone and serve as a source of calcium, phosphate, and magnesium ions for mineral homeostasis (Boskey & Robey 2013). In the context of *in vitro* differentiation, ascorbic acid (AA), β-glycerophosphate (β-GP) and Dexamethasone are well-established as being essential for differentiation and to induce mineralisation *in vitro* in rodent pre-osteoblastic cultures (Bellows et al. 1990) as well as in human MSC differentiation and mESCs/hESCs cultures (Buttery et al. 2001; Phillips et al. 2001; zur Nieden et al. 2003; Duplomb et al. 2007; Brown et al. 2009; Hwang et al. 2013). The majority of osteogenic differentiation experiments performed in the past have been conducted in serum-containing cultures.

However, in the serum-free culture system used here, the data presented until this point of the thesis, demonstrated that the use of these osteogenic factors had not triggered mineralisation during the differentiation phase, at least with the growth factor combinations and time course applied in these experiments. As this is a unique and novel serum-free system, it is possible that it lacks some osteoblast-specific inducing or cooperating factors for full osteoblast differentiation.

The model system used in this project made it possible to test several aspects of enhancing osteogenic differentiation towards mineralisation in a defined way and at a specific time points during the differentiation process. Thus, the next step was to test several additional conditions in an attempt to drive the ESC-derived osteoblasts to full maturity and mineralisation.

6.8 Methods

Briefly, the conditions tested were as follows: (1) extending the culture period to 4 weeks under the same media conditions; (2) supplementing the osteogenic media with batch-tested serum (FCS) or known osteogenic factors such as vitamin D3, Wnt3a, Hedgehog signalling agonists, low concentration of β-glycerophosphate; (3) culturing cells in FGF2 and BMP4 either separately or in combination; (4) culturing cells using a temporal FGF2 exposure; (5) re-plating differentiated osteoblasts in the presence of specific factors for an additional phase of differentiation/maturation under temporal FGF2 conditions. The effect of ROCK inhibition during the mesoderm enrichment phase (phase II) as well as during differentiation (phase III) was assessed in the context of the different culture conditions as indicated below.

6.9 Results

6.9.1 Extending the differentiation culture period

The original standard culture period used for osteogenic differentiation in phase III was 2 weeks (7-21d). It was therefore investigated whether simply extending the culture period for an additional 2 weeks (35d) would promote mineralisation. In the first instance, this was tested in the continuous presence of Y27632 as this had been shown in the first part of this chapter (partA), to maximise osteogenic differentiation and gene expression. Thus, cells were treated with Y27632 in phase II, and subsequently, in phase III, cells were cultured in osteogenic media containing Y27632 for a total of 28d (Fig. 6.6A). Following ALP and von Kossa staining, the results showed positive ALP staining (Fig. 6.6B), however, no signs of mineralisation were evident after von Kossa staining (Fig. 6.6C&D). These results indicated that simply extending the culture period with the standard osteogenic media conditions to a total of 35d was not sufficient to induce osteoblast mineralisation.

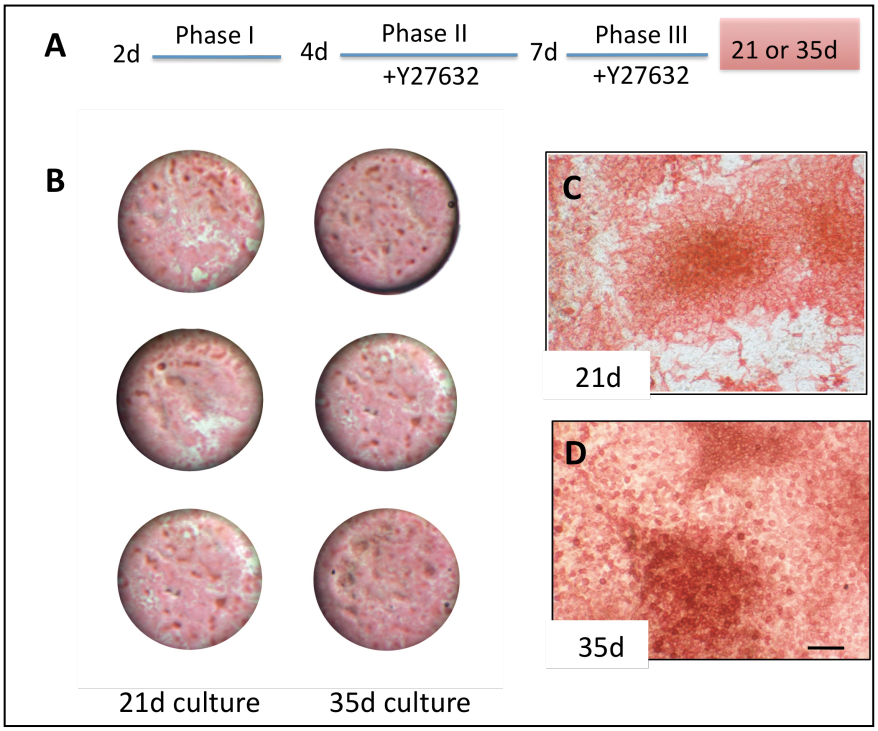


Figure 6.6: Extending the culture period (total 35d) does not induce osteoblast mineralisation.

A) A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. In phase III, cells were cultured in osteogenic media then fixed at 21d or 35 for ALP and von Kossa staining. B) Images of monolayers co-stained with ALP and von Kossa (triplicate wells). C,D) Representative images of monolayers after von Kossa staining showing 3D osteoblast nodules in both (C) 21d cultures and (D) 35d cultures. Scale bars: 100 µm.

6.9.2 Addition of batch tested serum (FCS) to the osteogenic culture media

The second approach undertaken to test the osteoblast mineralisation potential was to culture cells in a medium containing serum, acknowledging that serum-free cultures may have removed essential components required for mineralisation. As above, cells were cultured in phase II in the presence of Y27632, and in phase III cells were cultured in osteogenic media containing Y27632 for 28d, and additionally, FCS that was batch-tested for osteogenesis was added at three different concentrations, 10%, 1% and 0.1% (Fig. 6.7A).

The results revealed that cells cultured with 10% and 1% FCS were stimulated to proliferate more rapidly and reached confluence faster than cultures with 0.1% FCS or serum-free. During monolayer culture, 10% FCS showed an overgrowth of cells (not nodules) during the monolayer culture (Fig. 6.7B, arrow). While cultures in 0.1% FCS appeared to be able to differentiate compared to 10 and 1% serum cultures (Fig. 6.7H), although to a lesser extent than the serum-free control cultures, with no visible nodules present (Fig. 6.7D) as in the serum-free cultures (Fig. 6.7I). Long-term culture for 28d clearly did not enhance osteogenesis and mineralisation, and, in fact, addition of FCS markedly inhibited differentiation, which was evident in the FCS dose-dependent decrease in ALP staining compared to the control, serum-free culture (Fig. 6.7F-I). Together, these results implied that simply adding FCS was not sufficient to induce mineralisation; rather, serum negatively regulated osteogenic differentiation.

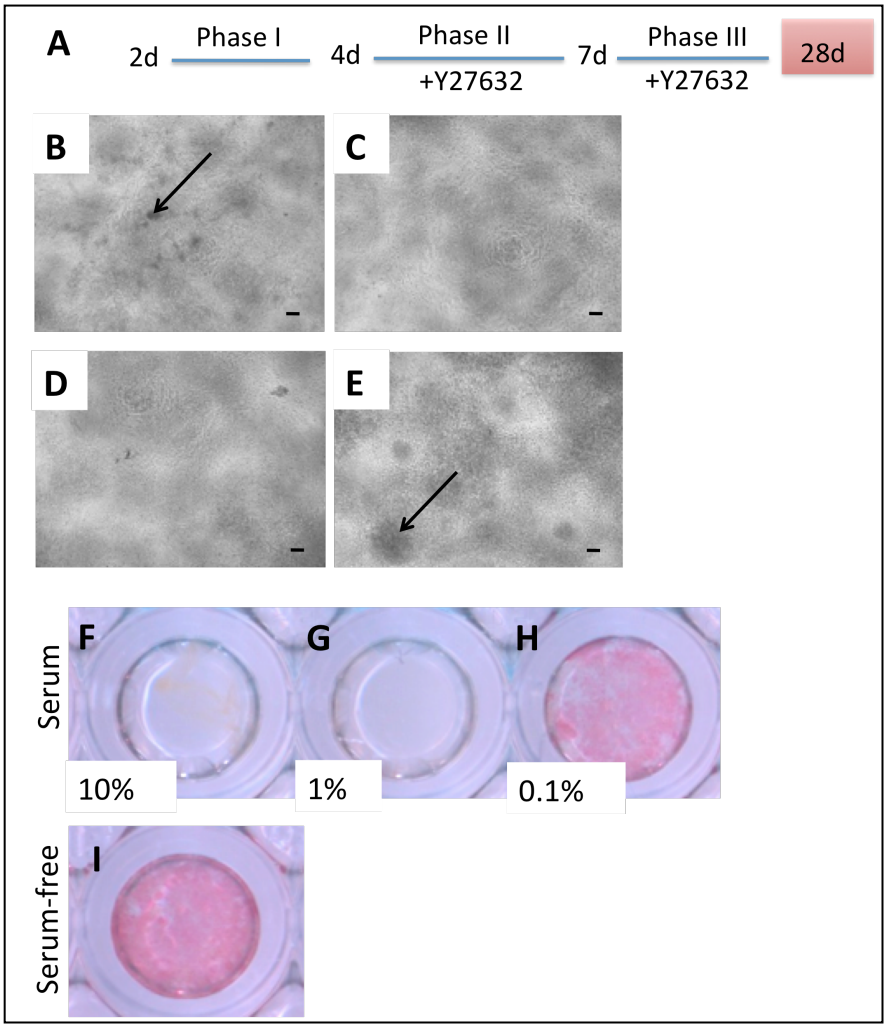


Figure 6.7: Effect of serum on osteogenic differentiation of ESC-derived mesoderm.

A) A differentiation scheme showing the timing of Y27632 addition to the culture media in phase II and III. In phase III, cells were cultured in osteogenic media in the absence and presence of batch tested FCS at 10%, 1% or 0.1%) for 3 weeks. B-E) Microscopic images of monolayers prior to staining showing; overgrowth of monolayer in 10% serum supplemented culture presented by dark areas (arrow) and clear nodule formation in serum-free cultures compared to 1% and 0.1% serum-supplemented cultures. F-I) Images of ALP stained monolayers. All images are from representative well from one experiment (triplicate wells). Scale bars: 100 µm.

6.9.3 Addition of osteogenic inducing factors to the osteogenic culture media

Another approach for testing osteoblast differentiation and mineralisation of osteoblasts was to take a candidate approach and test supplementation of the culture media during differentiation phase III with growth factors that had been shown to control bone formation. The effects of the following were tested: (1) Hedgehog signalling (Hh), a known inducer of osteoblast differentiation (Long et al. 2001b; Long et al. 2004; St-Jacques et al. 1999), by using a potent stimulator of Hh signalling, a Smoothened Agonist (SAG), which activates the Hh receptor Smoothened in a ligand-independent way (Chen et al. 2002); (b) Canonical Wnt signalling, which is known to control osteogenesis in both the embryonic and postnatal stages (Johnson et al. 2004; Hartmann 2009; Clevers & Nusse 2012). This was tested by the addition of Wnt3a to stimulate canonical Wnt signalling, hypothesising an induction in osteogenic differentiation; (3) Vitamin D_3, which has been shown to enhance the osteogenic differentiation and mineralisation of ESC-derived cultures in full FCS conditions (zur Nieden et al. 2003). This was tested by the addition of 1,25-dihydroxyvitamin D_3 ($VitD_3$); (4) a lower concentration of β-GP (2mM) instead of the commonly used dose of 10mM, which has been associated with poor osteoblast viability and dystrophic mineralisation (Orriss et al. 2007).

Cells were cultured in the presence of Y27632 in phase II, and subsequently in phase III in osteogenic media containing Y27632, and each of the test factors individually until d28 (Fig. 6.8A). The results revealed that after 28d of culture, all conditions displayed positive ALP staining (Fig. 6.8B-F), yet there were no signs of mineralisation following von Kossa staining (Fig. 6.8G-K). Interestingly, $VitD_3$ cultures showed a slight reduction in ALP staining compared to control cultures (Fig. 6.8D,I). These preliminary studies suggested that none of the factors tested seemed to stimulate further maturation and mineralisation under these experimental conditions and therefore no further characterisation was carried out at this time.

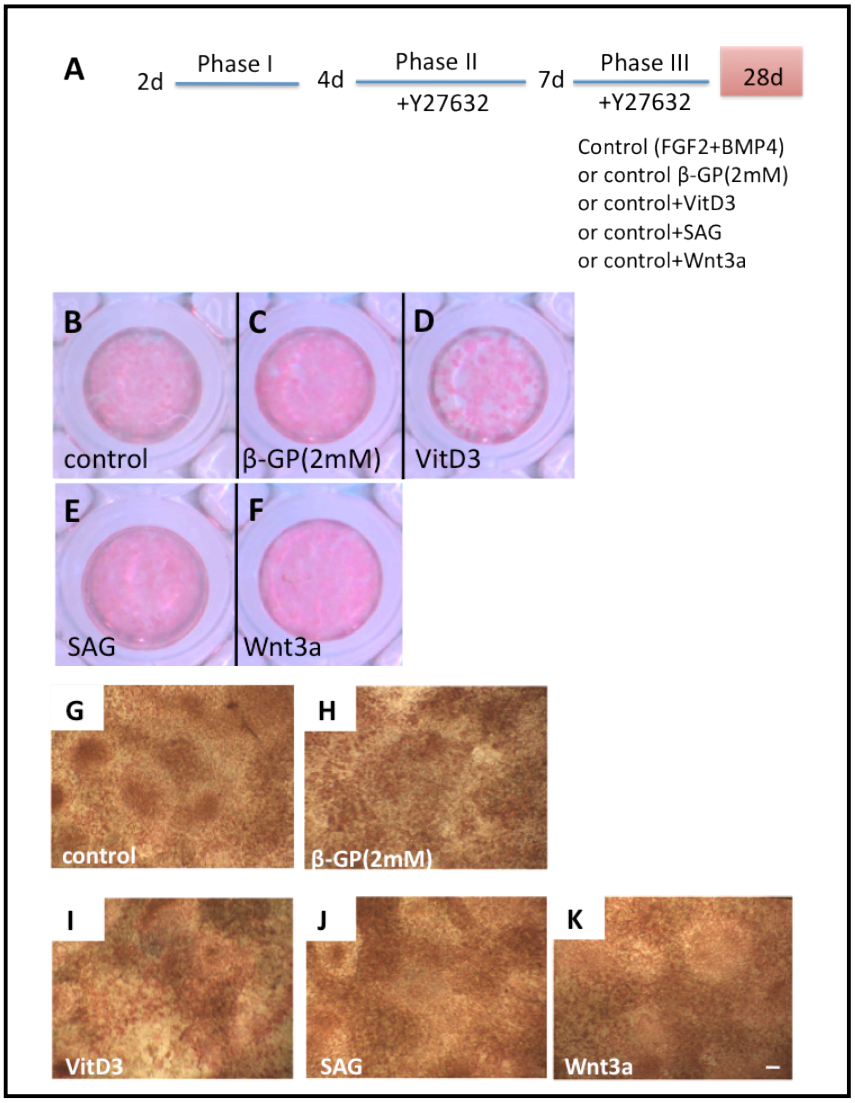


Figure 6.8: Effect of supplementing osteogenic media with known osteogenic inducing factors on osteoblast mineralisation.

A) A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. In phase III, cells were cultured in osteogenic media supplemented with one of the following osteogenic factors: β-GP (2mM), $VitD_3$ (10^{-8}M), SAG (100nM), Wnt3a (10ng/ml). Cultures were fixed after 3 weeks for staining. B-F) ALP staining of monolayers. G-K) Microscopic images of monolayers after ALP and von Kossa staining. All images are from a representative well from one experiment (triplicate wells). Scale bars: 100 µm.

6.9.4 Effect of FGF2 on osteogenic differentiation

Besides being important as a mitogenic factor, FGF2 signalling is also known to regulate osteoblast differentiation (Marie et al. 2012; Yu & Ornitz 2008; Dorey & Amaya 2010; Degnin et al. 2010). Notably, knock out studies have shown that loss of FGF2 causes inhibition of bone formation and reduction in bone mass (Montero et al. 2000). In addition, overexpression of FGF2 induces achondroplasia and shortening of long bone formation (Coffin et al. 1995). The mechanism of FGF2 in controlling osteogenesis can be multifaceted: the effects of FGF2 have been reported to either affect osteoblast proliferation or differentiation, depending on the developmental stage within the osteoblast lineage (Yu 2003; Fakhry et al. 2005). A large body of evidence supports the notion that FGF2 is important for proliferation, but can also inhibit osteoblast differentiation (Debiais & Hott 1998; Fakhry et al. 2005; Dailey et al. 2005).

In this project, the initial serum-free conditions for the differentiation phase (phase III) were carried out in the presence of both FGF2 and BMP in the culture media. It was thus questioned whether the presence of FGF2 could negatively affect osteoblast differentiation. Therefore it was decided to test the effects of culturing cells for 2 weeks in FGF2 and BMP4, either alone or in combination (Fig. 6.9A).

Results revealed that culturing cells only in FGF2 for 2 weeks inhibited osteogenesis compared to control, which was evident by the weak ALP staining (Fig. 6.9C,G,H). On the other hand, cells cultured only in BMP4 (Fig. 6.9D,I,J) presented similar ALP staining results to those that had been cultured in a combination of BMP4 and FGF2 (Fig. 6.9B,E,F). Furthermore, it was observed that FGF2 culture attained confluency earlier than BMP4 only cultures (data not shown), suggesting that FGF2 assisted in proliferation of the cells beyond those of BMP4 signalling.

The results further indicated that continuous FGF2 treatment inhibited osteogenic differentiation of ESCs. Nevertheless, it was still required at the early stages of differentiation of the cells in order for them to proliferate. Moreover, BMP4 separately or in conjunction with FGF2 did not induce osteoblast mineralisation.

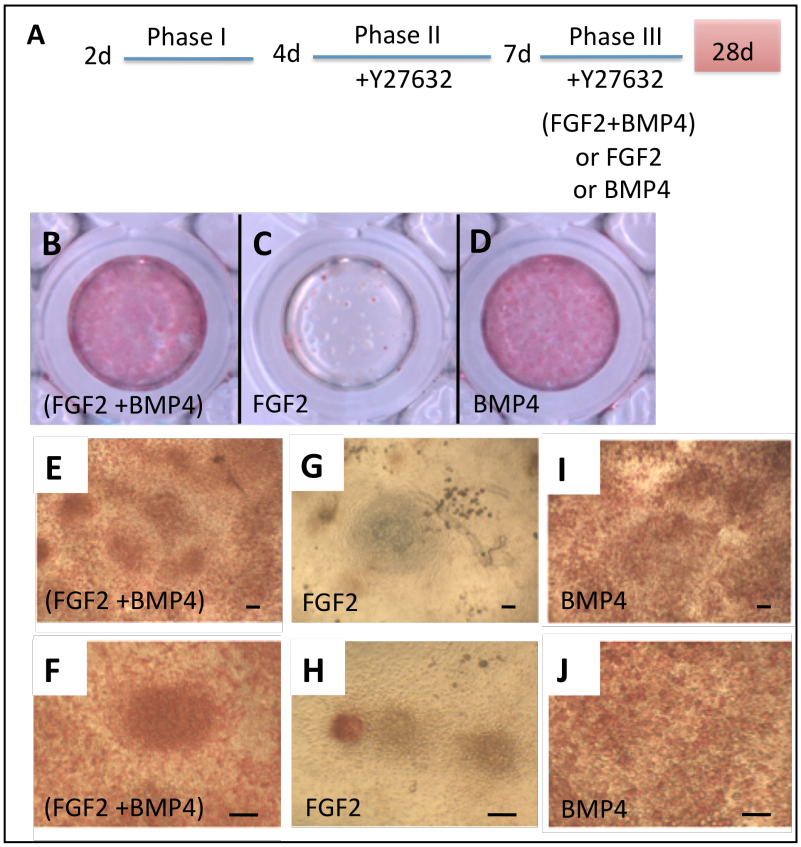


Figure 6.9: Effects of FGF2 and BMP4, either alone or in combination, on osteogenic differentiation.

A) A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. In phase III, cells were cultured in osteogenic media supplemented with either FGF2 or BMP4 alone, or in combination, then fixed after 3 weeks for staining. B-D) ALP staining of monolayers. E-J) Images of monolayers after ALP and von Kossa staining. All images are from a representative well from one experiment (triplicate wells). Scale bars: 100 µm.

6.10 Effect of FGF2 and BMP4 on primary calvaria osteoblast differentiation

Subsequently the role of FGF2 and BMP4 was investigated by using an independent differentiation system such as primary mouse calvaria, which is an established, well-known *in vitro* culture model for the differentiation and mineralisation of osteoblasts. Primary murine calvarial osteoblasts were cultured in differentiation media, which consisted of AA and β-GP, in both the presence and absence of batch-tested serum. The differentiation medium was then supplemented with BMP4 or a combination of BMP4 and FGF2 for 2 weeks.

In osteogenic media containing serum, BMP4-treated cultures stimulated both ALP staining and mineralisation by von Kossa staining (Fig. 6.10B). The addition of FGF2 to BMP4 cultures showed a marked inhibition of ALP activity and von Kossa staining compared to BMP4-treated cultures (Fig. 6.10C), indicating that FGF2 signalling inhibits both differentiation and mineralisation of primary calvarial osteoblasts.

A comparison with ESC cultures was attempted by differentiating primary osteoblasts in serum free conditions to interpret better the effects of BMP4 and FGF2. Whilst cells cultured in serum-free medium (control culture) failed to differentiate into ALP-positive cells (Fig. 6.10D). BMP4-treated cultures displayed some ALP staining, signifying that even in serum-free conditions, BMP4 alone can stimulate some ALP activity, albeit minimally (Fig. 6.10E). However, cultures treated with both FGF2 and BMP4 displayed a marked increase in ALP staining, but no mineralisation as assessed by von Kossa staining (Fig. 6.10F).

This suggests that FGF2 is very important for stimulating the differentiation of primary calvaria osteoblasts, although whether this is due to proliferation or survival is not known. Nevertheless, this provides independent evidence that FGF2 is required for survival and/or proliferation of osteogenic cells, as was also suggested for ESC-derived osteoblasts and indeed chondrocytes.

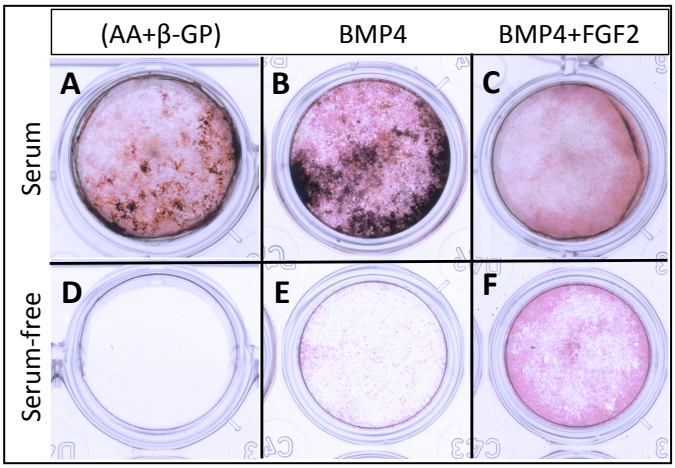


Figure 6.10: Effect of FGF2 and BMP4 on osteogenic differentiation of POB in serum and serum-free media.

Primary mouse calvarial osteoblasts were cultured in either serum (A-C) or serum- free media (D-F) supplemented with (AA and β-GP) in the presence of either (BMP4) or (BMP4 and FGF2) as indicated for 2 weeks. Cultures were then fixed and co-stained with ALP and von Kossa. All images are from a representative well from one experiment (triplicate wells).

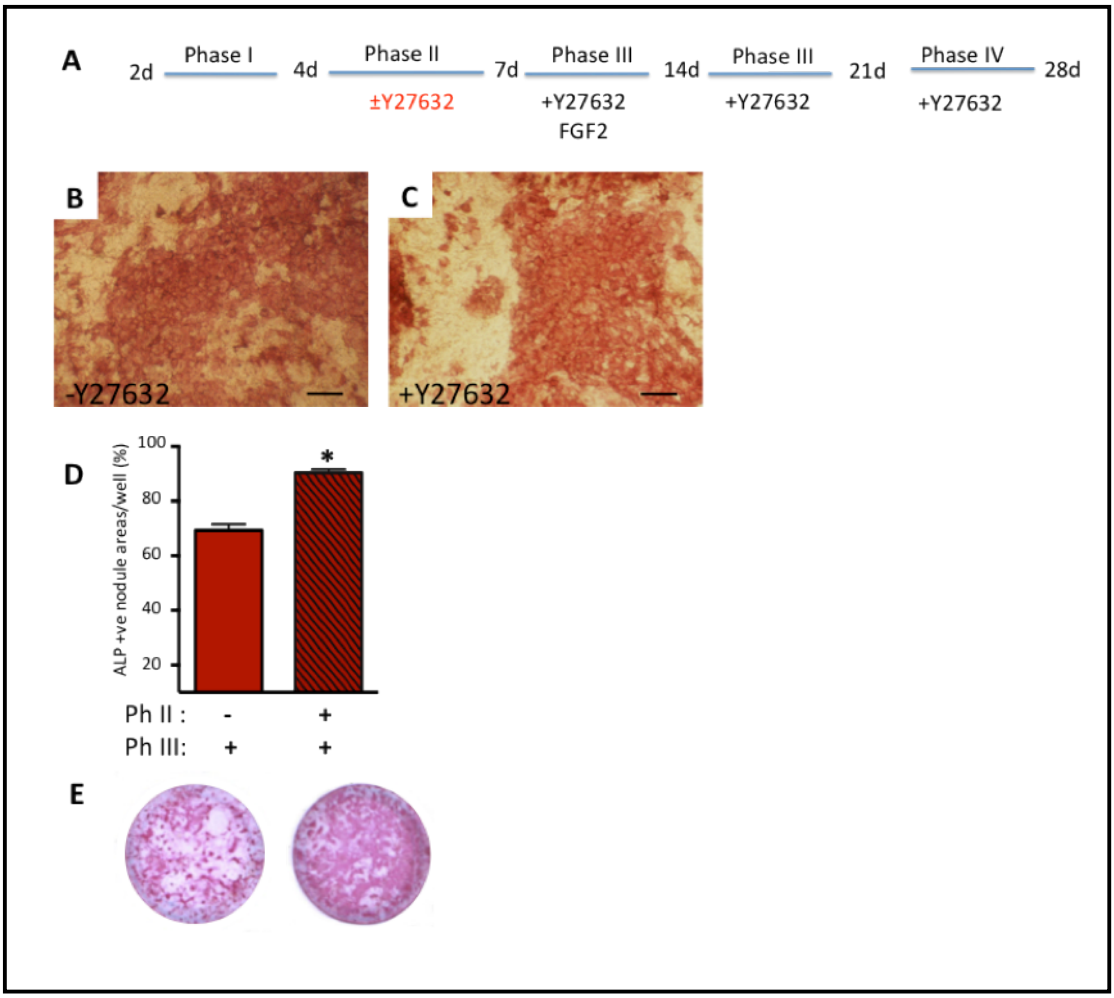
6.11 Long-term culture following re-plating of differentiated osteogenic cells

The results from the previous section were then used to probe whether re-plating end-stage cultures might provide a trigger for further differentiation and mineralisation. This was done under conditions of temporal instead of continuous FGF2 treatment, which was shown in the previous section to inhibit osteogenesis in a continuous exposure approach (Fig. 6.9 and 6.10). Therefore, cells treated in the absence or presence of Y27632 in phase II were cultured subsequently in Phase III in osteogenic media with Y27632, followed by removal of FGF2 after 1 week. After a further week, cells were dissociated and re-plated as single cells in osteogenic media containing Y27632 and BMP4, and this was designated Phase IV (Fig. 6.11A).

Morphological analysis demonstrated that osteogenic differentiation under a temporal FGF2 protocol generated osteoblast nodules (Fig. 6.11B&C), showing similar morphology to osteoblast cells differentiated under continuous FGF2 conditions (Fig. 6.11 B&C). Quantification of ALP positive nodule areas at 21d of differentiation showed that Y27632 treatment significantly increased the

percentage of ALP positive nodule areas compared to untreated cultures (Fig. 6.11D&E). Remarkably, following re-plating (phase IV), Y27632 pre-treated cultures began to show mineral deposits after reaching confluence when assessed by von Kossa staining at 28d (i.e. after one week of differentiation) (Fig.6.11H,J,L) compared to Y27632 not pre-treated cultures (Fig. 6.11G,I,K). These results implied that ROCK inhibition in phase II stimulated osteoblast mineralisation after re-plating when pre-cultured in temporal FGF2 conditions.

Therefore, it was established that differentiated osteoblast had the capacity to mineralise and attain a functional stage once cultured in temporal FGF2 and re-plated.



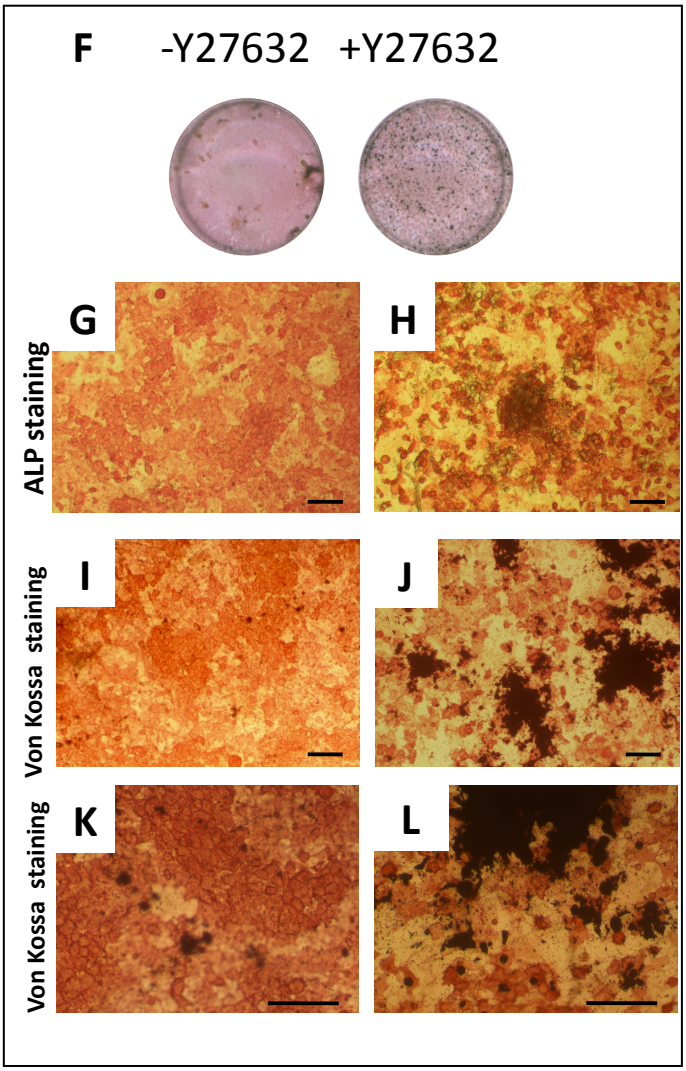


Figure 6.11: Effect of temporal FGF2 and ROCK inhibition on osteogenic differentiation after re-plating.
A) A differentiation scheme showing the timing of Y27632 addition to the culture media in phases II and III. In phase III, cells were cultured in osteogenic media supplemented with Y27632. FGF2 was removed from media at 14d. Some cultures were then fixed at 21d for staining and others were re-plated at 21d in a 48 well plate and fixed at 28d for staining. B,C) Microscopic images of ALP stained monolayers. D) Quantification ALP staining. E) ALP staining of monolayers. F) Co-staining of monolayer with ALP and von Kossa. F-H) Microscopic images of ALP stained monolayer, showing mineralisation in (+Y27632 cultures). I-L) Microscopic images of von Kossa stained monolayers, showing mineralisation in both (±Y27632 cultures). All images are from one of 2 representative experiments. Scale bars: 100 µm. Data indicate mean ± SD (triplicates) from a representative experiment of 2 independent experiments (paired Student t-test; * $p < 0.05$).

6.12 Discussion

In this section, it was demonstrated by ALP and von Kossa staining, that osteogenic cultures could produce mineralised matrix after further manipulation, mainly by extension of the culture period following re-plating of the cells. More importantly, this was enhanced by the inhibition of ROCK in phase II (mesoderm enrichment phase).

Mineralisation of osteoblast matrix has been demonstrated in mESC differentiation cultures (zur Nieden et al. 2003; Kawaguchi et al. 2005;Hwang et al., 2006) as well as in hESCs (Sottile et al. 2003; Vats et al. 2006; Shimko et al. 2004; Heng et al. 2004; Evans et al. 2009; Bielby et al. 2004; Handschel et al. 2010). All previous studies were undertaken in a serum containing osteogenic media. Furthermore, (Alfred et al. 2010) demonstrated mineralisation of mESC-derived osteoblast cultures in serum-free conditions, however, cells were exposed to serum, albeit in a bioreactor, at earlier stages of differentiation.

In this project, using the ESC/EB model, it was demonstrated that culturing ESC-derived mesoderm in osteogenic media for 2 weeks did not produce any signs of mineralisation based on morphology and von Kossa staining. Therefore, it was hypothesised that this could be due to several possibilities, such as factors related to exposure time, dose, growth factor interaction during culturing period, lack of mineralising inducing factors (serum, Wnt3a, SAG, VD_3), culture duration, mineralisation inhibitors, cell line, developmental stage of cells, and species type (Davis & Zur Nieden 2008; Lin & Hankenson 2011c). These factors will be discussed in the following sections.

Given the advantages of the serum-free culture system, it was possible to perform preliminary experiments to examine a few of these causes, including duration of culture period, culturing in the presence of well-known osteogenic inducing factors (Wnt3a, SAG, VD_3), low doses of β-GP and serum (FCS), FGF2 exposure time during culture and re-plating. On examination of these factors, it was possible to determine that ESC-derived mesoderm had the capacity to mineralise only when cultured in temporal FGF2 and then re-plated in osteogenic culture for 1 week.

Initially, osteogenic differentiation was carried out for two weeks during the differentiation phase (phase III; 7to21d). During this period, no mineralisation of the osteoblast nodules was observed. Previous studies of both murine and human ESCs showed mineralisation of osteoblast cultures within a period of 3 to 4 weeks in osteogenic media (zur Nieden et al. 2003; Bourne et al. 2004). Therefore, in this project, the culture period was then further lengthened by an additional two weeks, using the same osteogenic media (FGF2, BMP4, Dex, AA, β-GP). However, this still did not instigate mineralisation, and it is indeed possible that even longer culture periods beyond four weeks might be necessary, as the re-plating studies (as will be discussed later) seemed to provide conditions for allowing the mineralisation potential of the cultures to be realised.

Supplementing osteogenic media (in the continuous presence of FGF2 and BMP4) with the following osteogenic factors: Wnt3a, SAG, and VD_3, unfortunately, did not induce mineralisation and even showed a slight reduction in cultures supplemented with vitamin D_3 and FCS (10%, 1%). These unexpected negative ALP staining results could be due to the causes suggested earlier, including exposure time and dose of the osteogenic factors, in particular, the combination of FGF2 and BMP4, mineralisation inhibitors, developmental stage of cells, (Davis & Zur Nieden 2008; Lin & Hankenson 2011b). Zur Nieden et al. (2005) had demonstrated that osteogenic differentiation of ESCs was affected by both the exposure time and a combination of different osteogenic factors. They demonstrated that the time of BMP2 exposure influenced the osteogenic differentiation and that the combination of BMP2 with vitamin D_3 did not produce the same osteogenic effect as Vitamin D_3 alone. They also pointed out that Vitamin D_3 rescued the osteogenic phenotype when added to the culture at a later time point during the differentiation period. Vitamin D_3 was shown to induce mineralisation of ESC cultures during a period of (27-34d).

Additionally, β-GP is normally added to osteogenic media as a source for inorganic phosphate production. In previous studies, the β-GP concentration used normally in osteogenic media (10mM) has been shown to negatively affect mineralisation of pre-osteoblast cultures (Orriss et al. 2007). During the testing of our cultures, a 2mM β-GP dose and 10mM β-GP in the osteogenic media

produced no signs of osteoblast mineralisation. Therefore, at least in these culture conditions, the possibility of the effect of β-GP concentration on mineralisation of osteogenic cultures was ruled out.

The following osteogenic factors (Wnt3a, SAG, and vitamin D_3) were tested separately in an osteogenic media, however, the osteogenic media contained BMP4, Dex, β-GP, AA, and in the presence of Y27632. The presence of multiple factors in the media may have caused opposing interactions and affected the results. In addition, cultures were continuously exposed to FGF2; which had been demonstrated (as discussed later) to inhibit osteogenesis. Therefore, the combination of several factors at this particular time point and under the influence of ROCK inhibition may have induced opposing interactions.

During this project, ESC-derived mesoderm in osteogenic media containing AA, β-GP, Dex, BMP4 and FGF2 was used in continuous culture (i.e. for two weeks) as the basal culture conditions. Interestingly, however, it was proved that the continuous presence of FGF2 in osteogenic media inhibited osteogenesis when these cells were exposed to FGF2 for 2 weeks. A one-week pulse of FGF2 was adequate to induce osteogenesis and ROCK inhibition maximised the osteogenic differentiation.

The effect of temporal FGF on osteogenesis corresponds with other studies that support the belief that the effect of FGF2 on osteogenesis is not only dependent on the developmental stage of osteoblast, but is also dependent on the duration of exposure to FGF2. Prolonged FGF2 exposure inhibits osteoblast mineralisation, while short exposure was shown to have the reverse effect (Kalajzic, et al. 2003; Fakhry et al. 2005; Ling et al. 2006).

The effect of ROCK inhibition could be explained by its stimulatory effect on osteogenic precursors. Harmey et al. (2004) demonstrated that ROCK inhibition using Y27632 stimulates the osteogenic differentiation in primary mouse calvaria osteoblastc cultures by affecting the differentiation of osteoblast precursors rather than matrix deposition and mineralisation. This was evident by the increase in the number of ALP expressing cells leading to enhanced nodule formation and earlier mineralisation. Thus, the manifestation of mineralisation using our culture model could be because cells were exposed to temporal FGF2 for one week during phase III. Cells were then re-plated in

osteogenic media, which may have enhanced the proliferation potential of previously quiescent cells or stimulated the selective differentiation of pre-osteoblasts that was enhanced by ROCK inhibition.

The mineral deposits were localised to the osteoblast nodules, suggesting that this could be physiological instead of ectopic mineralisation caused by the addition of β-GP to the culture medium. To further establish that this mineralisation is due to a physiological process, it is suggested that further analysis is undertaken, using FTIR and TEM to study calcium and phosphate levels in the mineralisation process (Gentleman et al. 2009).

Taken together, it was established that osteoblast cultures could mature and mineralise in vitro. The unique and novel serum-free system used in this project provided the possibility to test several aspects of enhancing osteogenic differentiation towards mineralisation in a defined way and at a specific time and examine the role of ROCK inhibition. Based on morphology and von Kossa staining, it was demonstrated that re-plating cells after culturing in temporal FGF2 conditions was the only method that produced mineralisation of osteoblast and this was enhanced by the control of ROCK inhibition in phase II (mesoderm enrichment phase). Thus, these findings demonstrate that through the stage specific manipulation of appropriate signalling pathways it possible to generate distinct populations of mESC-derived osteoblasts that are able to generate growth plate-like cells *in vitro*, the *in vivo* potential of the generated cells (i.e. replating) would be interesting to evaluate and compare to the basal conditions (i.e. without plating) in future studies.

In conclusion, this is the first evidence showing osteogenic differentiation and maturation from ESC-derived mesoderm using this novel 3-phase, serum-free ESC/EB differentiation system used in this project. Understating the molecular mechanisms regulating this differentiation at the different development stages (i.e. mesoderm enrichment, differentiations, and replating phases) may shed light on the molecular role ROCK pathway possess in bone development and disease. Furthermore, access to a highly enriched population of osteoblasts at different developmental stags (i.e. progenitors or differentiated cells) will provide a basis for pursuing cell-based therapy for bone disease in cases of bone loss.

Chapter 7 *In vivo* evaluation of the chondrogenic and osteogenic potential of ESC-derived mesoderm: Effect of ROCK inhibition

7.1 Introduction

ESCs are considered an attractive cell source for studying the in *vitro* differentiation of specific cell types in a directed and controlled differentiation approach under appropriate manipulation of specific signalling pathways (Gadue et al. 2005; Murry & Keller 2008). The ability of ESCs to differentiate into any cell type from the three germ layers allows for the use of these cells for tissue replacement and regeneration therapy (Murry & Keller 2008; Irion et al. 2008). However, a major obstacle in the use of ESCs for this purpose is the ability to control and direct their differentiation efficiently so as to prevent potential undifferentiated ESCs from spontaneous differentiation and teratoma formation *in vivo* (Yamanaka et al. 2008).

In this project, as shown in previous chapters (chapters 4, 5, and 6), the 3 phases differentiation culture model directed the differentiation of ESC-derived mesoderm to cartilage and bone lineages *in vitro* that was accompanied by sequential expression of chondrogenic and osteogenic specific markers expression. However, whether these populations harbour any differentiation potential in an *in vivo* context is not known.

This chapter describes the preliminary proof-of-concept experiments that were performed to investigate whether ESC-derived chondrogenic/osteogenic cells could form tissue *in vivo*. Since the step-wise approach provides different stages of differentiation, the focus of this project was on two specific cell populations: (1) Cells derived directly from the mesoderm enrichment phase (phase II) that had already expressed cartilage and bone transcription factors, and (2) cells derived after the differentiation phase (phase III). In addition, based on the clear effect that ROCK inhibition had during both phases, further investigations were carried out to determine whether ROCK inhibition would influence the *in vivo* behaviour of these cells.

7.2 Methods

To investigate the *in vivo* potential of differentiated cells, cells were harvested from two developmental phases in our model: the mesoderm enrichment phase (phase II) and the monolayer differentiation phase (phase III). Firstly, at the mesoderm enrichment phase (phase II), cells were harvested from 7d cultures. These were then transplanted under the kidney capsule of adult immunocompromised (SCID) mice, in the form of aggregates or as a single cell suspension in matrigel. Matrigel was then transplanted without cells as a control. In addition, ROCK inhibitor-treated cultures were also investigated by the transplantation of Y27632-treated cultures. Secondly, at the differentiation phase (phase III), monolayers from 21d cultures, which gone through an osteogenic differentiation period in osteogenic media, were harvested and transplanted under the kidney capsule of SCID mice. ROCK inhibitor treated cultures were also investigated by specifically transplanting cells exposed to ROCK inhibitor at phase III only (7-21d) and cells exposed to ROCK inhibitor continuously (4-21d) in osteogenic media. For analysis, mice were sacrificed after periods of 1 and 3 weeks. Kidney samples were harvested, fixed, and processed for histochemical analysis using H & E with Alcian blue staining. All *in vivo* procedures were performed according to Home Office guidelines.

7.3 Results

7.3.1 Effect of ROCK inhibition on the *in vivo* potential of ESC-derived mesoderm at the mesoderm (phase II)

As described in chapter 3, the molecular analysis at the mesoderm enrichment phase (phase II) indicated that cells expressed key transcription factors for both chondrogenic (Sox9, Sox5) and osteogenic lineages (Runx2, Osx) at the end of the reaggregation culture period (4-7d). Under basal conditions, the expression of these genes is required for the initiation and differentiation of both cartilage and bone lineages (chapter 1, section 1.5). Therefore, to determine whether the early mesoderm-derived chondro-osteoprogenitor cells generated in phase II would have the capacity to generate cartilage and/or bone tissue *in vivo*, the generated aggregates were transplanted under the kidney capsule of SICD mice. Thus, cells were cultured for 3 days in FGF2 (phase II, 4-7d) and the generated aggregates were transplanted under the kidney capsule and left for 3 weeks (Fig. 7.1A).

The results demonstrated that the transplanted aggregates/cells had developed a tumour (Fig. 7.1B). Hematoxylin-eosin staining (H&E) analysis confirmed the formation of various tissues originating from the 3 germ layers (endoderm, ectoderm, and mesoderm), including gut-like epithelial tissue (gland), epidermal tissue (keratin), cartilage, and bone cell in the developed tumour on the site of transplantation in the kidney capsule (Fig. 7.1E&G). This suggested that cells still retained the pluripotent characteristic and had capacity to differentiate to cells from the three germ layers, indicating undifferentiated ESCs. Therefore, it is clear that cells generated at the mesoderm enrichment phase (phase II) of ESC/EB differentiation are tumorigenic and not fully differentiated.

As demonstrated in chapter 3, Y27632 treatment in the mesoderm enrichment phase (phase II: 4-7d), led to upregulation in the expression of both chondrogenic (Sox9 and Sox5) and osteogenic (Runx2 and Osx) gene expression. Therefore, priming the ESC-derived mesoderm cells differentiation towards chondrogenic and/or osteogenic lineages. In addition, other reports revealed that inhibition of ROCK signalling was linked to counteracting tumour progression and metastasis. ROCK signalling acts on actin cytoskeleton stabilisation and supporting actin-myosin contraction as well as the anti-angiogenic effect of ROCK inhibition (Itoh et al. 1999; Liu et al. 2009; Morgan-Fisher et al. 2013). Thus, understanding the role of ROCK inhibition effect on differentiation and anti-tumorigenic effect allowed to speculate whether ROCK inhibition would have a role in controlling the *in vivo* potential of 7d aggregates transplanted cells.

Therefore, to determine whether the early mesodermal-derived chondro-osteoprogenitor cells generated in ROCK inhibitor treated cultures, in phase II, would have the capacity to generate cartilage and/or bone tissue *in vivo*, the generated aggregates were transplanted under the kidney capsule of SICD mice. Hence, in phase II, cells were cultured in FGF2 in the presence and absence of Y27632. After 3 days in culture, at 7d, generated aggregates from both Y27632-treated and un-treated cultures were transplanted under the kidney capsule and left for 3 weeks (Fig. 7.1A).

Surprisingly, under macroscopic examination upon dissection and based on gross appearance, transplanted aggregates/cells from Y27632-treated (Fig. 7.1C) the tumours formed from Y27632-treated cultures appeared to be smaller

than tumours formed from Y27632 untreated cultures (n=4/5) (Fig. 7.1B&C), although the sizes were not quantified. No growths were observed in control matrigel grafts (Fig. 7.1D). Histological examination after H&E staining indicated the formation of cell types of the three germ layers in tumours derived from both Y27632-untreated (Fig. 7.1E&F) and Y27632-treated cultures (Fig. 7.1G&H). The tissues contained: gut-like epithelial tissue (e), epidermal tissue (keratin)(k), bone cell types (b), which are formed from the three germ layers, endoderm, ectoderm, and mesoderm, respectively. These results suggest that ROCK inhibition reduced the size of the developed tumour.

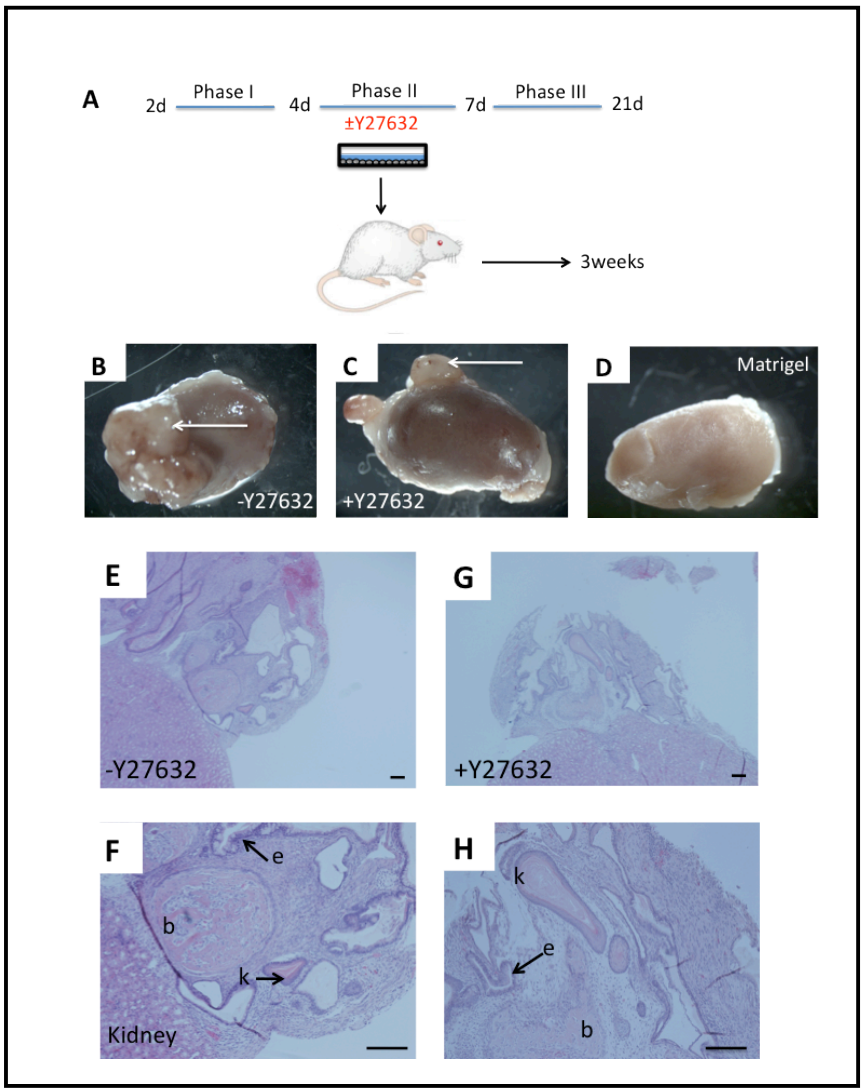


Figure 7.1: Effect of ROCK inhibition on the *in vivo* potential of cells generated from the mesoderm enrichment phase (phase II).

A) A differentiation scheme showing the timing at which cells were harvested for transplantation. In phase II, cells were cultured in the presence and absence of Y27632. Then after 3 days in culture (at 7d), cells were harvested and transplanted under the kidney capsule of adult immunocompromised (SCID) mice, in the form of aggregates or as single cells in matrigel suspension, and left *in situ* for a further 4 weeks and then harvested for analysis. Harvested kidneys from both Y27632-untreated (B) and Y27632-treated (C) transplanted cells showing teratoma lesions (arrows) that developed at the site of cell implantation. D) Image of control harvested kidney, where matrigel alone was transplanted, without cells. Histological section of tumour tissue harvested from kidney sample of transplanted Y27632-untreated monolayer (E&F) and Y27632-treated monolayer (G&H) stained with H&E and Alcian blue showing in arrows the differentiation of cell types from the three germ layers (ectoderm, mesoderm, and endoderm), including ectoderm component; epiderm tissue. Squamous epithelium showing a keratin deposition pattern (k), mesodermal components, and bone (b), and endoderm gut-like structures lined with mucinous epithelium (e) are indicated. Data are from a representative experiment from 5 independent experiments. Scale bars: 100um.

7.3.2 Effect of ROCK inhibition on the *in vivo* potential of cells generated from the monolayer differentiation phase (phase III)

Given that the transplantation of cells generated from the mesoderm enrichment phase (phase II) displayed teratoma formation, the next step was to examine the *in vivo* potential of cells, which had undergone the third phase of differentiation (phase III: 7-21d) in the 3-phase model. Cells pre-treated with Y27632 or not pre-treated were cultured for 2 weeks in osteogenic differentiation media in the presence of Y27632 (Fig. 7.2A). The monolayers were then transplanted under the kidney capsule of adult immunocompromised (SCID) mice for 1 to 3 weeks.

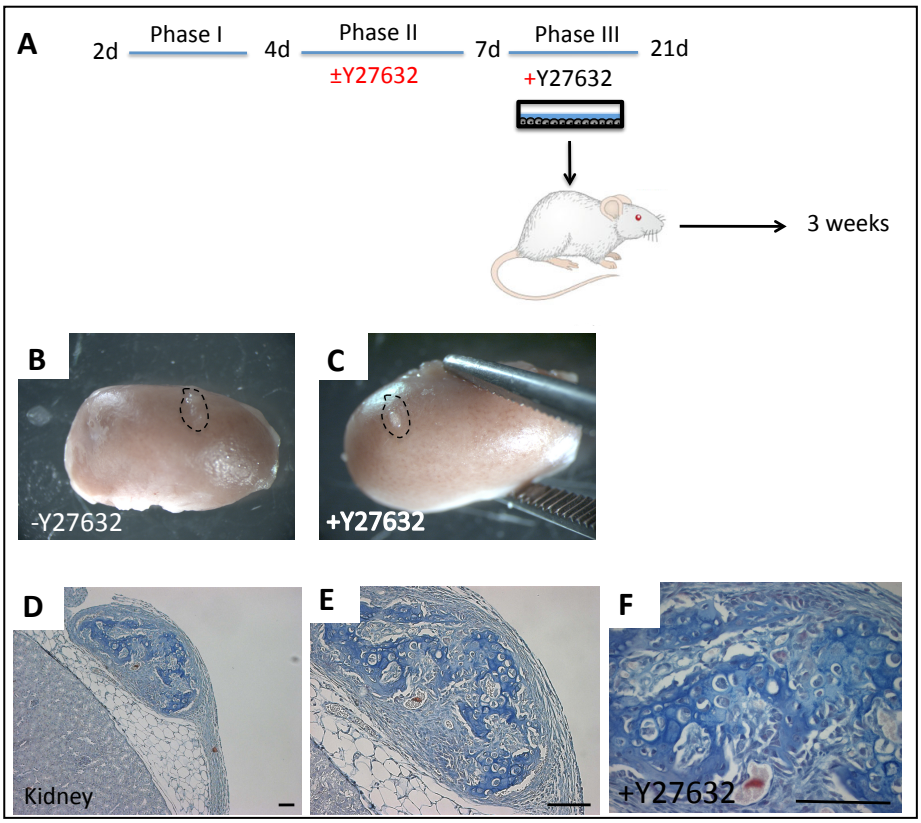
Macroscopic analysis upon dissection revealed small white lesions on the surface of the kidney samples at the site of transplantation that were hard in texture when palpated, suggesting possible mineralisation (Fig. 7.2B&C).

Histological analysis after H&E staining revealed the formation of bone tissue in the grafts (n=2). A time-course of bone development was carried out on grafts after only 1 week *in vivo*. Histological analysis showed the presence of cartilaginous tissue containing cells with typical round, chondrocyte morphology, which were surrounded by extracellular matrix that was stained intensely with Alcian blue in Y27632-treated samples (Fig. 7.2D-F).

Examination of the cartilage tissue after a further two weeks *in vivo* suggested that chondrocyte tissue was replaced by bone tissue in Y27632-treated samples (Fig. 7.2H). Therefore, transplanted phase III cells cultured in osteogenic media had undergone a temporal, endochondral ossification-type process *in vivo.* Furthermore, examination of samples (n=1) confirmed that the grafts from the Y27632-treated cultures appeared larger compared to the control i.e. untreated samples (Fig. 7.2I&J), although quantification was not performed. Significantly, no teratomas were observed in any of the grafts from 21d transplanted monolayers obtained from *in vitro* differentiation cultures (phase III: 7-21d).

Histological analysis of the formed bone revealed that it resembled natural trabecular bone structure comprising osteoblast-like cells lining bone surfaces (OB) and osteocytes (O) embedded within mineralised bone matrix (Fig.

7.2H&I). Some of the cells embedded within the bone matrix were enlarged and were associated with Alcian blue-stained matrix, resembling hypertrophic chondrocytes (HC), suggesting an endochondral ossification type of bone formation (Fig. 7.2H&I). In addition, the grafts were vascularised, which showed collections of blood cells (red blood cells (BC)) (Fig. 7.2I).



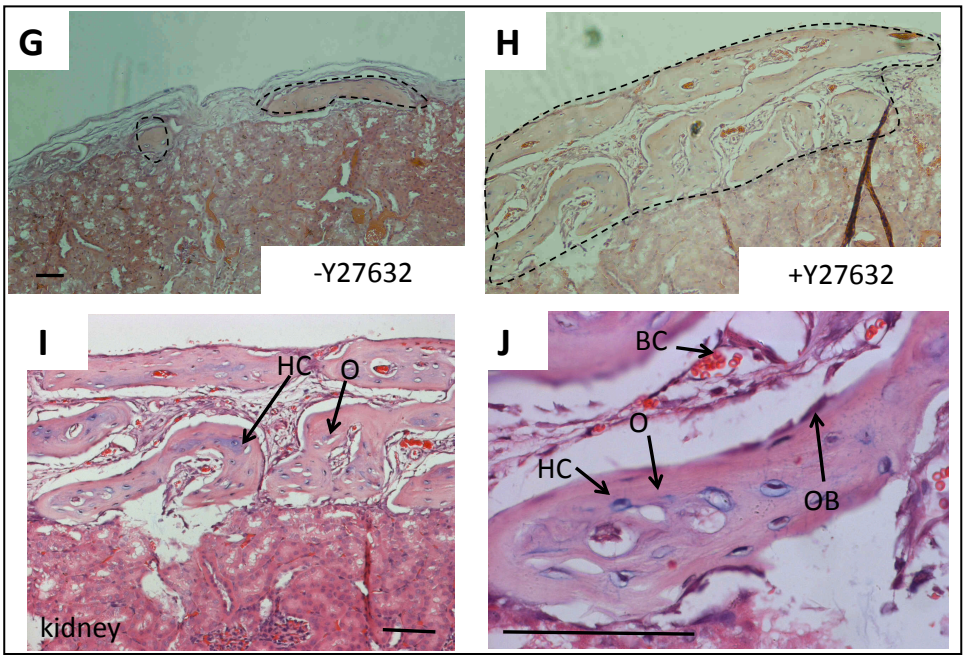


Figure 7.2: Effect of ROCK inhibition on the *in vivo* potential of cells generated from the differentiation phase (phase III).

A) A differentiation scheme showing the timing at which cells were harvested for transplantation. In phase III, cells were cultured in the presence and absence of Y27632 in osteogenic media. Then, after 2 weeks in culture (at 21d), cells were harvested and transplanted under the kidney capsule of adult immunocompromised mice (SCID), in the form of a monolayer, left *in situ* for 1-3 weeks and then harvested for analysis. Harvested kidneys from both Y27632-untreated (B) and Y27632-treated (C) transplanted cells showing white lesions that developed at the site of cell implantation (arrows). Histological sections of tissue stained with H&E and Alcian Blue showing the formation of chondrocyte cells with abundant Alcian Blue-positive matrix lying on top of kidney tissue formed after 1 week *in vivo* from Y27632-treated (D-F). After a 3-week in situ, section showing bone replacing cartilage tissue, resembling natural trabecular bone structure in Y27632-untreated monolayer (G) and Y27632-treated monolayer (H). I&J) Higher magnifications of the images in (H) showing a bone-like island compromising osteoblast-like cells lining bone surfaces (OB) and osteocytes (O) embedded within mineralised bone matrix, some of the cells embedded within the bone matrix were enlarged and were associated with Alcian blue-stained matrix, resembling hypertrophic chondrocytes (HC). Vascularisation showing collection of blood cells (red blood cells (BC) surrounding the bone structure. Scale bars: 100um.

7.4 Discussion

In this chapter, the preliminary proof-of-concept experiments proved the efficiency of the 3-phase, serum free ESC/EB differentiation *in vitro* culture model, whereby ESC-derived chondrogenic/osteogenic cells developed *in vitro*, specifically at the differentiation phase (phase III), which were cultured in osteogenic media, had the potential to form bone tissue *in vivo*. The formed bone tissue recapitulated embryonic development of the endochondral bone ossification process, which was characterised by the formation of a chondrocyte tissue, after 1 week *in vivo*, that was replaced by bone tissue after further 2 weeks.

Moreover, inhibition of ROCK signalling during the *in vitro* differentiation phase seemed to enhance *in vivo* osteogenic differentiation. Although several studies in the literature (chapter 1, section 1.10.2) have reported the efficiency of *in vitro* differentiation culture models for cartilage and/or bone in both mouse and human ESCs, as proven by expression analysis and flow cytometry analysis, yet fewer studies have carried out further functional analysis to investigate the *in vivo* potential of the generated cells. This emphasises the importance of translating the findings reported in chapters 4-6 of this project, to an *in vivo* context, which provides evidence for the *in vivo* functionality of *in vitro* differentiated cells.

The advantages provided by the step-wise, 3-developmental phase culture model used in this project allowed examination of the *in vivo* potential of different populations generated, at different time points during differentiation, i.e. early and late stages of differentiation. Herein, the two time points tested were the mesoderm enrichment phase (phase II) and the differentiation phase (phase III), 7d and 21d cultures, respectively.

The molecular profile results discussed in chapter 3 proved that during the mesoderm enrichment phase (phase II: 4-7d), the cells were primed towards differentiation towards both chondrogenic and osteogenic lineages. The 3-day FGF2 treatment (reaggregation) upregulated the key transcription factors for both cartilage and bone lineages (Sox9 and Sox5) and (Runx2 and Osx), respectively. In addition, molecular profiles of gene expression of pluripotent markers showed a reduction in Oct-4 expression in two consecutive phases

ofESC differentiation (i.e. mesoderm induction and enrichment phase), suggesting progression of cell differentiation and gradual reduction in the pluripotency genes characteristic of ESCs. However, transplanted reaggregates/cells from 7d cultures demonstrated tumorigenic potential, suggesting that the population still contained some undifferentiated ESCs. Thus, the differentiation conditions during the mesoderm enrichment phase (phase II) proved to be too heterogeneous and sub-optimal for cells to form cartilage or bone tissue *in vivo*.

Surprisingly, although ROCK inhibitor treated-cells demonstrated tumorigenic capacity, ROCK inhibition seemed to reduce the ESC capacity of the transplanted cells, as evidenced by gross examination of the tumour (n=4/5). Thus, ROCK inhibition might influence differentiation of ESCs. Several studies have reported the *in vivo* effects of ROCK inhibition on tumorigenesis whereby ROCK inhibition using Y27632 prevented tumour invasion and metastasis, explained by counteracting activation of ROCK, which acts on actin cytoskeleton stabilisation and supporting actin-myosin contraction as well as through the anti-angiogenic effect of ROCK inhibition (Croft et al. 2004; Somlyo et al. 2003; Rath & Olson 2012). Further quantification is needed of the size of tumours generated from ROCK inhibitor treated and untreated tumour samples.

Subsequently, *in vitro* functional analysis of cells generated at 21d of culture during the differentiation phase (phase III) was conducted to direct ESCs towards both chondrogenic and osteogenic differentiation. Chondrogenic differentiation was proven to acquire hypertrophic chondrocyte characteristics as demonstrated in (chapters 4 and 5). On the other hand, osteogenic differentiation exhibited mineralisation after further manipulation of the culture conditions and prolonged culturing (i.e. after temporal FGF2 exposure and re-plating, defined as phase IV: 21-28d) as shown in chapter 6. Therefore, given that the differentiation culture model used in this project is a serum-free model, this suggests that optimal conditions had not yet been met in the *in vitro* cultures. Thus, cells from the osteogenic media, at 21d of culture, were selected in order to prove their functionality in an *in vivo* environment. Transplantation of these cells clearly demonstrated ectopic bone formation and a lack of tumour formation in both the absence and presence of the ROCK inhibitor cultures. Moreover, analysis of samples from one experiment

suggested that bone formation in ROCK inhibited samples appeared to be enhanced; as this was not measured, further experimentation and quantification may add valuable information to the anabolic effect seen with ROCK inhibition treatment. Even though, these results support the osteogenic and mineralisation potential of the cells demonstrated in chapter 6, whereby exposure to ROCK inhibition, specifically in phase III, enhanced osteogenic differentiation and advanced it towards mineralisation after prolonged culturing (chapter 6).

Previous *in vivo* studies findings support the anabolic effect of ROCK. Yoshikawa et al. (2009) demonstrated in mice that continuous delivery of ROCK inhibition using Y27632 enhanced ectopic bone formation when induced by rhBMP-2 impregnated into an atelocollagen carrier. Therefore, based on these studies and our preliminary study it could be speculated that ROCK inhibition during *in vivo* differentiation of cells might have advanced the cells for further differentiation down the osteogenic pathway. A methodical time-course study of xenograft development from ESC-derived osteogenic cells would address this.

Interestingly, the boney tissue formed *in vivo* demonstrated a bone formation that was reminiscent of an endochondral ossification process, as cells formed chondrogenic tissue early, after 1 week of transplantation, which was then replaced by bone after 3 weeks. Therefore, the differentiation culture model used in this project was proven to provide completely defined conditions for osteogenic differentiation, both *in vitro* and *in vivo*. This is perhaps expected, as the cultures that were grafted were a mixed cell population containing cells with both chondrogenic and osteogenic potential (see also chapters 4-6). Further studies of more enriched or purified chondrogenic or osteogenic populations, either through further optimisation of growth factor conditions, or through cell sorting, will be necessary to identify their *in vivo* potential. Nevertheless, this suggests application of an endochondral ossification approach for bone regeneration (Craft et al. 2013; Yang et al. 2013), whereby an intermediate cartilage formation stage (cartilage phase) is required to prime cells for bone formation. The rationale behind this approach is that chondrocytes are able to survive with limited nutrition and oxygen. Secondly, they are able to secrete vascular endothelial growth factor (VEGF) in the hypertrophic stage, which is beneficial for blood vessel growth and attraction of osteoblast precursors for the initiation of the bone formation stage (Pfander and

Gelse 2007). Thus, mixed chondro-osteoprogenitor populations still provide a suitable source for regenerative therapy applications. Indeed, the mixed chondrogenic and osteogenic potentials of transplanted cells has been proven in other studies to be beneficial for bone regeneration, based on both ESCs (Jukes et al. 2010) and MSCs (Pelttari et al. 2006; Tasso et al. 2009; Tortelli et al. 2010; Scotti et al. 2010; Farrell et al. 2009; Farrell et al. 2011).

In this project, there are several limitations in terms of graft versus host contribution. Tracing the fate of the grafted cells was not performed thus the possibility of host cell contribution to the xenograft growth was difficult to assess. As the cells used for this project were unlabelled male ESCs, one option was to trace the localisation of male Y chromosome sequences by using Immunofluorescence *in situ* hybridization (ImmunoFISH). However, the SCID adult mice used in this study were male. Another better option, which will be pursued in future experiments, is the use of a fluorochrome (eg. GFP)-based fluorescent protein labelled reporter cell line, where it would be possible to trace all ESC derivatives, or trace specifically chondrogenic or osteogenic derivatives.

Furthermore, the anabolic effect seen in ROCK inhibited samples was encouraging in that *in vivo* results matched the *in vitro* results; however, further analysis of a larger sample size and measuring bone volume would be beneficial. In addition, better optimisation is required to dissect the *in vivo* potential of chondrogenesis versus osteogenesis samples. Regarding the graft site, the *in vivo* experiments were carried out in the kidney capsule. Kidney capsule implantation, although technically challenging, has been shown to be a good model for testing ectopic bone formation, It promotes robust bone growth by providing supraphysiologic blood and nutrient resources (Scott et al. 2012). Other implantation models, such as intramuscular implantation, have been used for BMP *in vivo* bone formation confirmation experiments. With intramuscular transplantation it may be difficult to distinguish host from graft cells due to the mixture with muscle satellite cells, specifically if cells are not labelled. However, it proved to be a good model for testing cartilage phenotype stability (Dell'Accio et al. 2001; Eltawil et al. 2009).

In summary, *in vivo* ectopic bone formation results confirmed the osteogenic differentiation results obtained *in vitro* in osteogenic media over 2 weeks. The bone tissue was preceded by chondrocyte tissue formation, thus mimicking the temporal embryonic development of endochondral ossification. In addition, inhibition of ROCK signalling seemed to enhance bone formation. These results could suggest a promising source of generating osteogenic progenitor cells. Further investigation is necessary of the chondrogenic cells differentiated from *in vivo* cultures and optimisation of *in vivo* conditions for better tracing of graft cells.

In conclusion, further understanding of the effect of ROCK inhibition at the cellular and molecular levels could still be promising. The regenerative potential of ROCK inhibition could be advanced in the development of future treatments and translation of the step-wise ESC/EB culture model to human ESC or iPSC model systems.

Chapter 8 General discussion and future work

The aim of this project was to investigate the role of ROCK signalling via the use of Y27632 ROCK inhibitor, on cartilage and bone lineage specification and differentiation by using a novel 3-phase, serum free, ESC/EB differentiation model system. This project established that ROCK inhibition differentially regulates ESC-derived mesoderm differentiation to chondrocytes and osteoblast in a phase-specific manner i.e mesoderm enrichment (phase II) and differentiation phases (phase III). This was evident based on results from histochemical staining quantification and lineage-specific gene expression analysis.

8.1 The mesoderm enrichment phase (phase II)

It has been established that ROCK signalling inhibition may modulate the differentiation of intermediate progenitor populations, sub-mesoderm and chondro-osteoprogenitor populations. The findings in chapter 3 demonstrate that the inhibition of ROCK signalling upregulates the expression of both chondrogenic and osteogenic key transcription factors simultaneously, suggesting the commitment of ESCs towards the chondrogenic and osteogenic lineages (Fig. 3.13 4d vs 7d+Y). This role of ROCK inhibition at an intermediate developmental stage, such as the mesoderm enrichment phase in ESCs is done here for the first time and is unique to this project.

Interestingly, compared to control cultures, ROCK inhibition during the mesoderm enrichment phase (phase II) proved to differentially regulate the differentiation of ESC-derived mesoderm towards a distinctive chondro-osteoprogenitor population. Expression of Sox9 and Osx was downregulated and Sox5 expression was upregulated with no effect on Runx2 expression (Fig. 3.13, 7d-Y vs 7d+Y). The outcome of this differential effect of ROCK inhibition was further investigated by functional *in vitro* differentiation analysis demonstrated in chapter 4 (Fig. 4.3, -/- vs +/-) for chondrogenic differentiation and chapter 6 (Fig. 6.3, -/- vs +/-) for osteogenic differentiation. Taken together, the results establish that exposure to ROCK inhibitor at the mesoderm enrichment phase (-/- vs +/-) differentially induces the chondrogenic over the osteogenic lineage as presented in the proposed model (Figure 8.1).

Furthermore, the differential effect of ROCK inhibition on chondrogenic and osteogenic key transcription factors expression might be linked to the ROCK inhibition effect on sub-mesoderm genes profile demonstrated in chapter 3 (Fig. 3.11 and Fig. 3.12, 7d-Y vs 7d+Y). The results show that inhibition of ROCK signalling differentially regulates the expression of paraxial and lateral plate mesoderm transcription factors, suggesting that ROCK inhibition might be directing ESC differentiation towards a distinctive paraxial and lateral plate sub-mesoderm populations compared to ROCK inhibitor untreated cultures (7d-Y vs 7d+Y). Therefore, supporting the differential effect on cartilage and bone gene expression profile.

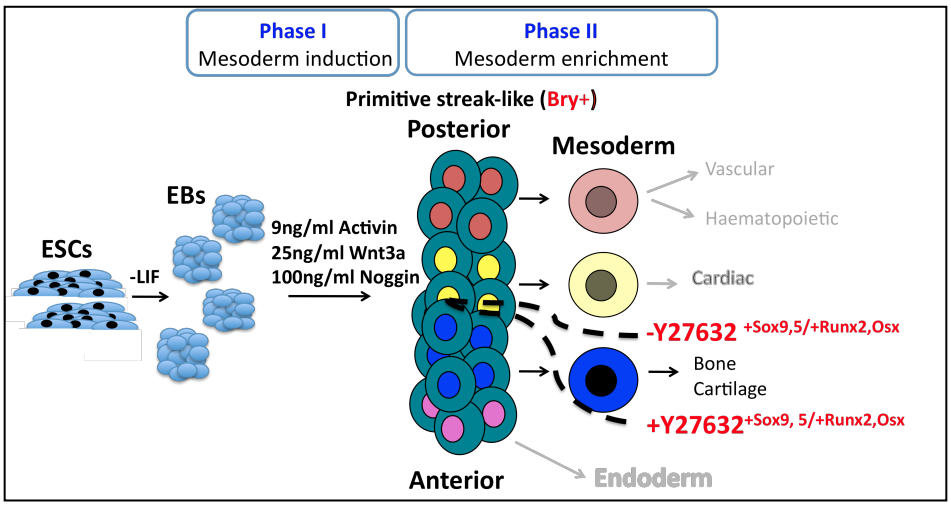


Figure 8.1: A summary of the effects of the ROCK inhibitor on chondrogenic and osteogenic commitment of ESCs at the mesoderm enrichment phase (phase II).

At the mesoderm enrichment phase (phase II), the exposure to ROCK inhibitor (+Y27632) upregulated the expression of key transcription factors for both chondrogenic (Sox9 and Sox5) and osteogenic (Runx2 and Osx) as the control (-Y27632) (data in figure. 3.13). However, ROCK inhibition differentially regulated the mentioned transcription factors (data in figure. 3.13), suggesting the generation of a distinctive chondrogenic and osteogenic progenitor population, a “chondro-osteoprogenitor population “. Diagram modified from Keller 2005.

8.2 The differentiation phase (phase III)

In this project it was established, without cell sorting and purification approaches, that ROCK signalling modulates the specification and differentiation of cartilage and bone from ESC-derived mesoderm. The findings in chapters 4 and 6 clearly demonstrate that the exposure to ROCK inhibitor at the mesoderm enrichment phase is the key stage for the anabolic effect on cartilage (-/- vs +/-) (Fig. 4.7B).

On the other hand, the exposure to ROCK inhibitor at the differentiation phase proved to be the key stage for the anabolic effect on bone (-/- vs -/+) (Fig. 6.5). Furthermore, the long-term exposure to ROCK inhibitor further enhanced the differentiation of both cartilage and bone (+/+) compared to temporal ROCK inhibition (-/+) and basal condition (-/-) (Fig. 4.7, 5.2, and 6.11), respectively, evident by the increase in the percentage of Alcian blue or ALP positive nodule area and gene expression analysis. Taken together, these results established that the effect of ROCK inhibition on chondrogenic and osteogenic differentiation is a phase-specific dependent effect (Fig. 8.2).

8.2.1 Short-term exposure to ROCK inhibitor

In the context of chondrogenic differentiation, findings in chapter 4 demonstrate that Y27632 pre-treatment in phase II (-/- vs +/-) (Fig. 4.3), stimulates a 3-fold increase in the percentage of Alcian blue positive nodule and upregulates cartilage-specific gene markers expression at 14d of differentiation, suggesting the positive influence of inhibiting ROCK signalling on cartilage differentiation. These stimulatory results of ROCK inhibition agree with previous studies conducted on primary cell cultures and ATDC5 cell lines (Wang et al. 2004; Woods et al. 2005; Tew & Hardingham 2006). On the contrary, the change in cell culture model i.e. micromass cultures showed a negative response to ROCK inhibition (Woods & Beier 2006), which may indicate that the developmental stage of cell differentiation as well as the culture model may influence the response of cells to ROCK signalling.

In the context of osteogenic differentiation, findings in chapter 6, demonstrate that Y27632 pre-treatment in phase II (-/- vs +/-) (Fig. 6.3 and 6.5), inhibits

osteogenic differentiation evident by a significant decrease in the percentage of ALP positive nodule area and bone-specific gene markers expression. Several studies have suggested that the developmental state of the cells does influence the response to ROCK inhibition (McBeath et al. 2004; Arnsdorf et al. 2009). McBeath et al. (2004) reported that ROCK inhibition via Y27632 and constitutively active ROCK overexpression in cells decreased osteogenic differentiation in hMSCs cultures. Similarly, Arnsdorf et al. (2009) showed similar results in murine C3H10T1/2 cultures upon treatment with Y27632. These results have led to the question of whether cells at the differentiation phase III (7-21d) could be at a MSC–like state.

Indeed, it would be interesting to further investigate the expression of the standard MSC surface markers in ESC-derived cells, and investigate the potential of the cells to differentiate into other cell types of mesodermal/MSC origin, such as adipocytes or myocytes (Kopher et al. 2010; Harkness et al. 2011; Mahmood et al. 2012). Moreover, further characterisation of the generated cell population is also necessary as investigating the kinetics of osteogenic gene marker expression and measuring ALP activity during the differentiation process would allow for further confirmation of the inhibitory effect of ROCK inhibition on osteogenesis at the differentiation phase.

Taken together, the findings suggest that ROCK inhibition at the mesoderm enrichment phase is key in positively regulating chondrogenesis while negatively regulating osteogenesis at the molecular and cellular levels. Further analysis of the kinetics of gene expression at different time points during the differentiation phase (phase III) will be beneficial to investigate further and understand the effect of ROCK in a dynamic manner.

8.2.2 Long-term exposure to ROCK inhibitor

8.2.3 Chondrogenesis

Long-term ROCK inhibition (-/+ vs +/+) (Fig. 4.5 and 4.7) enriched chondrogenic differentiation as observed by a 7.2-fold increase in Alcian blue positive nodule areas as well as promoting hypertrophic chondrocyte differentiation as evidenced by cell morphology and Col X expression. Interestingly, further manipulation of the culture conditions by culturing cells in temporal FGF2

demonstrated to act synergistically with ROCK inhibition and further enhancement of the hypertrophic phenotype (Fig. 5.2 and 5.3).

Additionally, it was demonstrated for the first time that ROCK inhibition enhances non-hypertrophic chondrocyte differentiation in short-term cultures when implementing GDF5 instead of BMP4 signalling. The effect of GDF5 on ESC–derived mesoderm chondrocyte differentiation have been proposed by Craft et al. (2013) where the switch between BMP and GDF5 signalling influenced the *in vitro* differentiation of chondrocyte to either hypertrophic or non-hypertrophic phenotype. Therefore, ROCK signalling may play an important role in mesoderm specification towards hypertrophic and non-hypertrophic chondrocytes and this has implications for generating stable articular cartilage for potential osteoarthritis applications. To this end, I have been involved in preliminary studies that are show marked differences in the ability of BMP4- and GDF5-stimulated chondrocytes to differentiate *in vitro* in specific hydrogel formulations (Toh et al. 2009) (Kania, Bukhary, Grigoriadis).

8.2.4 Osteogenesis

Findings in chapter 4, 5 and 6, strongly suggest that long-term ROCK inhibition (-/+ vs +/+) manipulation increases osteogenic differentiation by 1.4-fold. Compared to temporal ROCK inhibition and basal conditions, respectively. In addition, it directs the maturation of chondrocytes (Fig. 5.2 and 5.3) and osteoblasts (Fig. 6.11), possibly mimicking endochondral ossification. The ability of the osteoblasts to mature further and attain full mineralisation *in vitro* was demonstrated in chapter 6, part B. This is the first evidence showing osteogenic differentiation and maturation from ESC-derived mesoderm using this novel 3-phase, serum-free ESC/EB differentiation system used in this project. A preliminary experiment was conducted to investigate the potential of osteoblasts to reach to functional osteoblasts i.e. mineralisation. The findings described in chapter 6, part B (Fig. 6.11), demonstrate that ROCK inhibition in the mesoderm enrichment phase (phase II) enhances the production of mineralized matrix of osteoblasts evident by ALP and von Kossa staining. This was demonstrated after modification of the culture conditions by culturing cells in temporal FGF2 and prolonged culturing followed by replating differentiated cells (21d cells). Therefore, these findings suggest that ROCK inhibition,

specifically in the mesoderm enrichment phase (phase II: 4-7d), is the driving force for osteoblast mineralisation.

In the context of osteogenic differentiation, I have performed preliminary experiments showing that ESCs cultured in osteogenic media could attach and differentiate on osteoconductive hydroxyapatite scaffolds (Daculsi et al. 2013) (data not shown). The findings suggested that application of ESC-derived osteogenic progenitors could be influential for the future application in bone regeneration or repair of bone defects. The utilisation of different biomaterials have been broadly investigated by others in the context of ESC-derived chondrogenic and/or osteogenic progenitor cells, in order to direct cell differentiation for tissue regeneration and the generation of functional tissue *in vivo* using mESC, hESCs and iPSCs (Hwang et al. 2008; Scott et al. 2012; Craft et al. 2013; de Peppo et al. 2013; Teng 2013). However, by using the ROCK inhibitor it could provide additional mechanistic insights into whether ROCK inhibition would advance the established methods of tissue engineering.

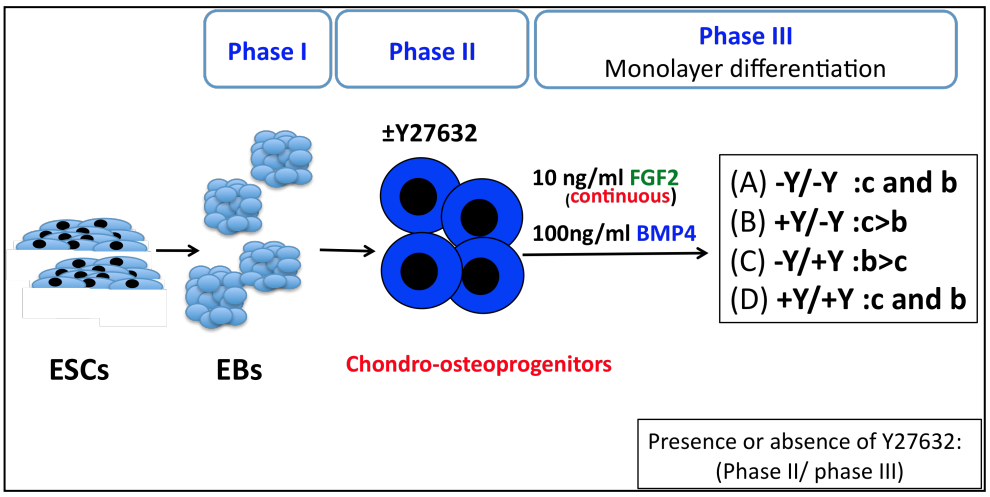


Figure 8.2: A summary of phase-specific effect of ROCK signalling inhibition via Y27632 (Y) differentially regulates chondrogenic and osteogenic differentiation of ESC-derived mesoderm during the monolayer differentiation phase (phase III)

At the monolayer differentiation phase (phase III), the exposure to the ROCK inhibitor either in a short-term exposure (+/-) and (-/+) or a long-term exposure (+/+) modulates the differentiation of a putative chondro-osteoprogenitor population as marked by quantification of Alcian blue for chondrocytes (c) and ALP for osteoblasts (b), and lineage-specific marker expression. (A) Basal conditions (-/-) were shown to differentiate ESC-derived mesoderm to both lineages. (B) Short-term exposure to Y27632 at phase II (+/-) showed preferential differentiation towards chondrogenic over the osteogenic lineage, while (C) short-term exposure to Y27632 at phase III (-/+) showed a preferential differentiation towards osteogenic over the chondrogenic lineage (-/+). (D) Long-term exposure to Y27632 at phases II and III (+/+) showed differentiation to both lineages, however, long-term exposure to ROCK inhibition directed the differentiation towards hypertrophic chondrocytes (data in figure 4.7, 5.2, and 5.3) and mineralised osteoblast differentiation (data in figure. 6.11). Diagram modified from Keller 2005.

8.3 *In vivo* potential of ESC-derived chondrocytes and osteoblasts

In chapter 7, the *in vivo* potential of the differentiated cells confirmed the endochondral ossification potential of the differentiated cells *in vitro* (Fig. 7.2). The implanted differentiated cells from a 21d culture underwent a chondrogenic phase of differentiation in the first week of transplantation, which was replaced completely by bone tissue after an additional 2 weeks *in vivo.* The bone formation potential seemed to be enhanced by ROCK inhibition, however, this needs further confirmation on a larger number of samples and quantification analysis of bone tissue. In addition, investigating the *in vivo* potential of cells derived from the other temporal ROCK inhibited cultures (i.e +/-) would be interesting to further analyse whether the differentiated cells would undergo similar differentiation to those tested in chapter 7 (i.e. -/+ and +/+).

The evidence on whether ectopic bone formation originates from the graft itself will require further analysis. One approach could be by the use of female SCID mice as hosts, and investigate the presence of the Y-chromosome marker since the CCE cell line is derived from male embryos. Alternatively, the advancement in the use of reporter ESC lines specifically for cartilage or bone lineages would provide genetic labeling for better tracing cell lineages. Such an approach would be beneficial to analyse the effects of ROCK inhibition, for example, to investigate whether the temporal inhibition of ROCK during the mesoderm enrichment phase (+/-) would give rise to progenitors that have either endochondral ossification or stable articular cartilage potentials.

8.4 Conclusion and future work

For the mesoderm enrichment phase, accessing intermediate populations of chondrocyte and osteoblast precursors by using the ROCK inhibitor (Y27632) in the novel 3 phase, ESC/EB differentiation culture model, will permit for further understanding of the molecular mechanisms regulating cartilage and bone differentiation and the downstream events regulated by ROCK signalling. Furthermore, analysis by quantification of the percentage of progenitors expressing cartilage and bone specific markers such as Sox9 and Runx2 by flow cytometry, or using specific ESCs with GFP knocked into specific transcription factor loci will provide further data on the efficiency of the differentiation protocol and investigate whether ROCK influences this process. Moreover, further analysis of the kinetics of cartilage and bone markers gene expression at different time points during the mesoderm enrichment phase (phase II: 4-7d) would be helpful in understanding the maturity stage of the cells before differentiating them in phase III as well as the downstream signalling factors controlling cartilage and bone differentiation. In this regard, we have begun to identify ROCK inhibitor-dependent global changes in gene expression through microarray analysis during this important mesoderm enrichment phase, and it will be interesting to identify novel pathways that might contribute to establishing the chondrogenic and osteogenic lineages.

Whereas in the differentiation phase, through the phase-specific manipulation of ROCK signalling pathways it was possible to generate distinct populations of ESC-derived chondrocytes and osteoblasts at different developmental stages. In addition further manipulation of other signalling pathways such as FGF2 and BMP signalling allowed for the generation of growth plate-like cells or articular cartilage-like cells *in vitro*. Ultimately, this will provide insights into further understanding and characterisation of embryonic development of both mesoderm cartilage and bone progenitors, in particular in response to ROCK signalling manipulation.

In the last few years, several studies have demonstrated that Rho GTPase siganlling, including ROCK signalling, can affect cell differentiation through the interaction with the cell cytoskeleton, ECM (i.e. in detecting matrix stiffness) and cell shape (Shih et al. 2011; Mih et al. 2012). In addition, ROCK inhibitors, specifically Y27632, are now routinely implemented for culturing hESC cultures (Claassen et al. 2009; Cortes et al. 2009; Gauthaman et al. 2010; Lai et al. 2010; Watanabe et al. 2007). However, the exact mechanisms of ROCK inhibitor-dependent effects, in particular on cartilage and bone differentiation is not clear and further investigation is necessary to shed light on the downstream signalling pathways or regulatory genes that are responsive to ROCK signalling. Ultimately, these will identify novel pathways that might contribute to regulating chondrogenic and osteogenic lineages, especially at the early developmental stages represented by the novel ESC differentiation model system used in this project.

The translation of this novel ESC differentiation model system from mouse ESCs to human ESCs or iPSC model systems would be an essential next step for this research. The advantage being is the accessibility it offers to progenitor cells, either chondrogenic or osteogenic, specifically through ROCK signalling manipulation may provide the generation of distinctive chondrogenic and/or osteogenic progenitors for tissue replacement therapy, for the treatment of bone and/or cartilage defects such as osteoporosis and osteoarthritis. Therefore, provide a biological means for further understanding the mechanisms of cartilage and bone development in healthy and diseased cells using a cell-based approach, and complemented by animal models.

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