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Commitment and Transdifferentiation of Adult Progenitor Cells: the Contributing Pathways

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COMMITMENT AND TRANSDIFFERENTIATION OF ADULT PROGENITOR CELLS: THE CONTRIBUTING PATHWAYS

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Thesis submitted to the
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Abstract

C2C12 murine progenitors are a model for transdifferentiation. This project investigated signaling events that regulate this process in culture by monitoring specific markers for transdifferentiation (alkaline phosphatase [ALP] and myogenin for the osteoblastic and myoblaststic fates respectively) in the presence of inducers and inhibitors. Antibodies specific for proteins implicated in signaling were used to monitor the differentiation process.

BMP-2 addition caused transdifferentiation to the osteoblastic fate except for a small fraction of cells that may represent the so-called MyoD negative cells or the side population (SP). The timing of short term commitment of C2C12 to osteoblastic transdifferentiation was between 6 to 24h under our culture conditions. BMP-2-induced ALP activity increase and the repression of myogenin expression were not simultaneous. Differentiation as measured by ALP activity may be dependent on the interaction of BMP-2/Smad, p38 MAPK and TGF-β1 pathways. Smads 1, 5 and/or 8 were almost always found under their phosphorylated form (pSmads) and thus almost invariably located in nuclei suggesting fast nucleoplasmic shuttling from the cytoplasm and nuclear retention of the phosphorylated forms. Phosphorylated forms were also found in the nucleus under proliferative conditions without exogenous BMP-2. In this case, it is suggested that this phosphorylation of Smads may be triggered by the reported autocrine secretion of TGF-β by the C2C12 cells or by the TGF-β present in the culture serum.

Résumé

Les progéniteurs murins C2C12 servent de modèle pour la transdifférenciation. Ce projet a pour but d'étudier les voies du signalement cellulaire qui régissent la transdifférenciation en culture *in vitro* en présence d'inducteurs et d'inhibiteurs. Les marqueurs alcaline phosphatase (ALP) et myogénine ont permis de suivre l'évolution respective des phénotypes ostéoblastiques et myoblastiques après induction par le morphogène BMP-2. On a utilisé des anticorps spécifiques des protéines impliquées dans le signalement pour leur étude au cours du déroulement du processus de transdifférenciation.

L'addition de BMP-2 a induit la transdifférenciation des cellules vers le phénotype ostéoblastique excepté pour une petite fraction qui pourrait représenter les cellules souches résidentes embryonnaires MyoD négatives ou les cellules SP. Le moment où les progeniteurs C2C12 sont commis à un phénotype ostéoblastique se situe entre 6 et 24h dans nos conditions de culture. L'induction de ALP et la répression de la myogénine ne sont pas simultanées. L'évolution de l'ALP durant la différenciation semble dépendre de l'interaction entre BMP-2/ Smad, p38 MAPK et les voies de signalement du TGF-β.

Les proteines Smads 1, 5 et 8 sont presque toujours sous forme phosphorylées (pSmads) et se trouvent donc invariablement localisées dans le noyau ce qui suggère un va et vient rapide du cytoplasme vers le noyau suivi d'une longue rétention dans le noyau. Les noyaux des cellules en proliferation non soumises à l'induction par le BMP2 contiennent aussi les pSmads. L'hypothèse est émise, selon laquelle la phosphorylation des Smads serait induite, dans ce cas, en réponse à la sécrétion autocrine de TGF-β par les cellules C2C12 ou par le TGF-β présent dans le sérum du milieu de culture.

Dedication

Especially dedicated to: Alicias, Betica, Gaito, Martha, Consuelo, Juandi, Orlando, Pachito, Lolas, Clodoveo and Jenny.

Also to: Carlos A., Henry, Pedrito, Ulises, Lucero, Teli, Nivia, Tita, Nieves, Estebanes, Fabio, Fercha, Flechas, Alejandra, Milton., Nasly, Alcaravan, Mansa, Moni, Maito, Camis, Alvarin, Jairos, Alita, Nadia, AlbertoV., Socios, Manuelito, Melbia, Baquero, Maria T, Miguel, Diane, Bryson, Magdalena, Fernando R., Gaby, Alfonsito L, Stellita, Herreras, Dot, AlvaroW, Diegos, Regine, Leito, Oscar, Miladin, Costenito, Andres, Marie C., Jean-Paul, Ana Maria, Brent, Bernardo, Abaunzita, Ferris, Jean-Claire, Charlebois, Hung, Aliaga, Majeau, Shaida, Sean, Papi, Angelita, Martha Cecilia, Delio, Oliverio, Fabiola, Daniel Augusto, Morales, Moreno, Forero-Carrero, Marth'higuera, Briseida, Myriam R., los primitos, Ascencion y Ligia Bonza.

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ActR-I	-	Activin receptor type I
AHZ	-	Beta-alanyl-L-histidinato zinc
ALK	-	Activin receptor-like kinase
ALP	-	Alkaline phosphatase
AMHR	-	Anti Mullerian hormone receptor
ATCC	-	American type culture collection
BCIP	-	5-bromo-4-chloro-3-indolylphosphate
BMP	-	Bone mrphogenetic protein
BMPR-I	-	BMP receptor type I
BMPR-II	-	BMP type II receptor
BSA	-	Bovine Serum Albumin
Co-Smad	-	Common Smad
CtBP	-	C-terminal binding protein
DAPI	-	4'-6-Diamidino-2-phenylindole
DiI	-	1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanineperchlorate
DMEM	-	Dulbecco's Modified Eagle's Medium
DNA	-	Deoxyribonucleic acid
dpc	-	Days post coitum
EDTA	-	Ethylenediaminetetraacetic acid
ERK	-	Extracellular Signal-regulated kinase
ES	-	Embryonic stem cells
FBS	-	Fetal bovine serum

GDF5	-	Growth and differentiation factor 5
HEPES	-	N-2-Hydroxyethylpiperazine-N′-2-ethane-sulfonic acid
HRP	-	Horseradish peroxide
Id1	-	Inhibitor of DNA binding 1 protein
I-Smads	-	Interference or inhibitory Smads
IgG	-	Immunoglobulin G
MAPK	-	Mitogen-activated protein kinases
MFH-1	-	mesenchyme forkhead-1
MH1	-	MAD protein homology
mRNA	-	Messenger ribonucleic acid
α-MEM	-	Alpha minimum essential medium
MIS	-	Mullerian inhibiting substances
Myc	-	Myelocytomatosis viral oncogene
NBT	-	Nitro Blue Tetrazolium
NC	-	Neural crest
NCSCs	-	Neural crest stem cells
NES	-	Nuclear export signal
NP40	-	Nonidet P-40
PAGE	-	Polyacrylamide gel electrophoresis
PAI-1	-	Plasminogen activator inhibitor type 1
PBS	-	Phosphate buffered saline
PMSF	-	Phenylmethylsulfonyl
pNP	-	Nitrophenylphosphate disodium
rhBMP-2	-	Recombinant human bone morphogenetic protein 2

RIPA	-	Radioimmunoprecipitation assay
R-Smads	-	Receptor regulated Smads
Runx2	-	Runt related transcription factor 2
SAD	-	Smad activation domain
SDS	-	Sodium dodecyl sulphate
SDS-PAGE	-	Sodium dodecyl sulphate poloyacrylamide gel
SSC	-	Standard sodium citrate buffer
TAK	-	Transforming growth factor-beta-activated kinase
TGF-β	-	Transforming growth factor beta
TRβ –I orII	-	TGF-β typeI or II receptor
TBS	-	Tris-buffered saline
TBST	-	Tris-buffered saline with Tween-20
Tris	-	2-amino-2-hydroxymethyl-1,3-propanediol
UV	-	Ultra violet
WNT	-	Wingless/Int
XIAP	-	X-linked Inhibitor of Apoptosis

Chapter 1
Introduction and Literature Review

1.1 Origin of Osteoblasts and Bone during Embryonic Development

During mammalian embryonic development, a single cell divides repeatedly to produce the blastula and then later the blastocyst that consists of an inner cell mass or embryoblast and an outer cell mass or trophoblast. In the developing embryo, four essential processes (cell proliferation, cell specialization, cell interaction and cell movement) give rise to the many cell phenotypes that contribute to a unique final pattern of spectacular complexity and precision.

The cells derived from the blastocyst inner cell mass give origin to the three germ layers: the ectoderm which is the precursor of the epidermis and nervous system; the endoderm that will give rise to the gut and its appendages and the mesoderm that gives rise to connective tissue, bone (of interest in this project), cartilage, and the circulatory and lymphatic systems. During development, embryonic stem cells and other cells progressively lose some of their plasticity when going from totipotent to unipotent. is the process that leads a cell to a single cell lineage. In the embryo, bone develops from osteoblasts (a cell that makes bone by producing a matrix that then becomes mineralized) found in both the mesoderm and ectoderm-derived neural crest cells.

Regardless of the embryonic lineage (mesodermic or Neural Crest origin), ossification or bone formation occurs in two different modes: Through intramembranous direct ossification and through endochondral ossification.

1.2 Origin of Osteoblast Precursors in Post Natal and Adult Organisms

In post natal organisms, mesenchymal stem cells were first reported to be located in the bone marrow (Becker *et al.*, 1963) but are now known to have a much wider distribution. These cells have since been isolated from other tissues such as umbilical chord blood, adipose tissue, rodent skin, and human hair follicules (Toma *et al.*, 2001; Yu *et al.*, 2006; Lee *et al.*, 2007; Gimble *et al.*, 2007) where they may remain dormant until they are needed to replace old or damaged tissue. Mesenchymal stem cells found in adults are progenitor cells that exhibit limited plasticity and are sometimes limited in self-renewal.

Stem cells of mesodermal origin whether found in embryo, fetus or adults have in common the potential to differentiate into chondroblasts, osteoblasts, adipocytes or myoblasts (Pittenger *et al.*, 1999; Dennis *et al.*, 1999; Mendes *et al.*, 2005). Adult mesnchymal progenitors can commit and differentiate to either one of these determined cell types under specific environmental conditions. These progenitors can also undergo transdifferentiation, a process that takes place when a cell phenotype changes fate. For example, the mesenchymal precursor C2C12 cell that was studied in this thesis can undergo both of these processes. It can commit to the myoblastic lineage and can transdifferentiate from its myoblastic fate to osteogenic (Katagiri *et al.*, 1994), chondrogenic (Takada *et al.*, 2004) or adipogenic fates (Yeow *et al.*, 2001) see Figure 1.1.

The two main molecular mechanisms underlying transdifferentiation are commitment (which can entail both transcriptional repression and induction of new gene sets) and maturation/differentiation. Both these processes are driven by cellular signaling. Cellular signaling is a process defined as the conversion of a signal into cellular responses that allow the cells to adapt to the demands of their micro-environment (Faeder *et al.*, 2005). During cellular

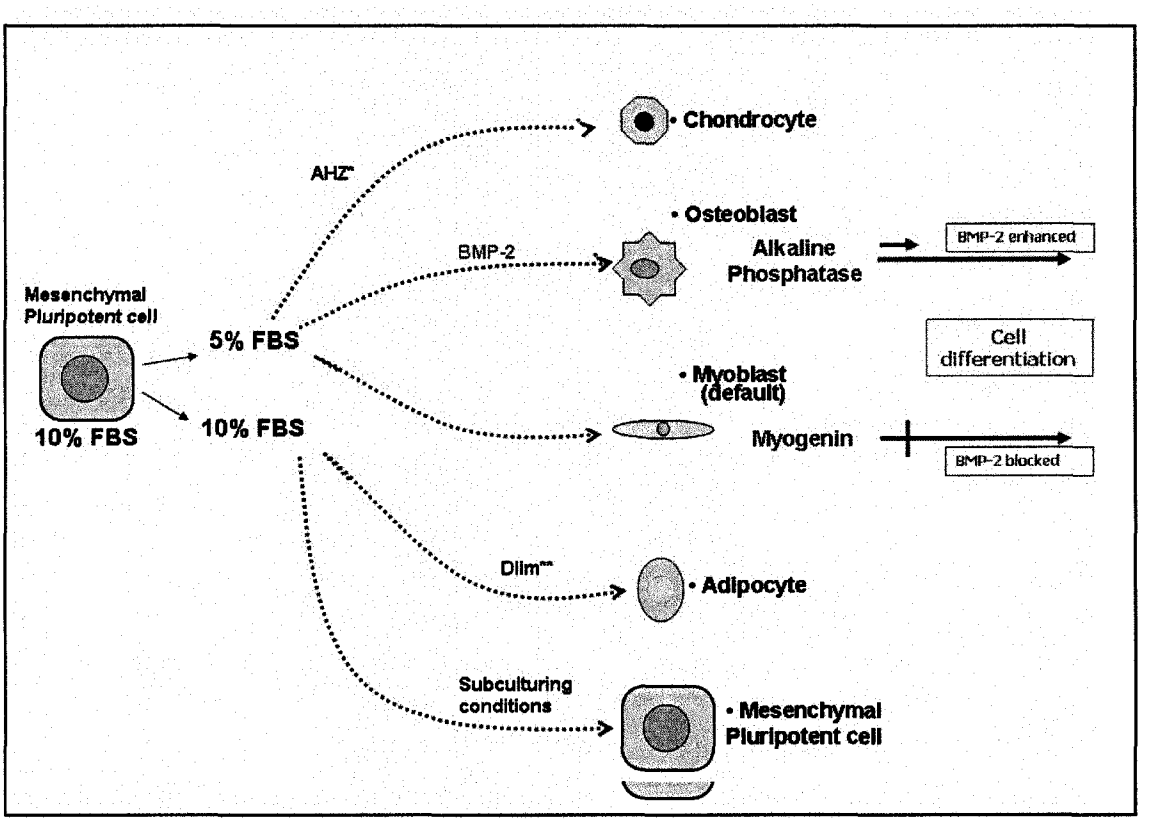


Figure 1.1 C2C12 lineage committment via transdifferentiation. The C2C12 cell line can commit/transdifferentiate to different cell lineages depending on the culture conditions. *AHZ: beta-alanyl-L-histidinato zinc. **DIim: Dexamethasone Insulin3-isobutil-1-methylxanthine

signaling, extracellular molecules or autocrine factors like growth factors or hormones act as ligands for matching transmembrane receptors, which transduce the signal to the nucleus where it induces a transcriptional response. Cellular signaling should be understood as a web in which different induced pathways interrelate and evolve. The involved transmembrane receptor is usually a protein kinase (enzyme that catalyzes the transfer of a phosphate group) that elicits a cascade of successive phosphorylation steps thus activating a series of downstream substrates. The last molecule in the cascade is transferred to the nucleus and triggers transcription of a new set of genes. For example the Ras-Raf-Mek1/2, ERK1/2 cascade that acts as molecular switch to control a wide range of essential biochemical pathways in eukaryotic cells. This cascade is important in cell proliferation and the coupling of intracellular signal transduction pathways (Bar-Sagi and Hall, 2000).

In another less common type of signaling the ligand binds to the receptor activating it. The receptor activation leads to homo or hetero dimerization of its substrates that are further transferred to the nucleus where they trigger transcription of a new set of genes. This is the case for the JAK/STAT pathway that is the principal signaling mechanism for a wide array of cytokines and growth factors (Rawlings *et al.*, 2004) and the Smads pathway important in cellular processes such as commitment, differentiation and apoptosis. Smads play a major role in osteoblast commitment and differentiation.

1.3 *In vitro* Multipotent Progenitor Cell Model

When committed, stem cells or progenitors from an adult organism can be cultured *in vitro* or as xenografts (cells transplanted to another species). With adequate growth specific factors, they progress into a preferred phenotype that may be depicted as their "default state". This is true for the satellite cells (the committed stem cells of the skeleton muscles) used in this

study (Alexakis *et al.*, 2007). Upon exposure to specific growth factors such as the ones belonging to the transforming growth factor beta (TGF-β) superfamily of growth and differentiation factors, the progenitors of skeletal muscles can switch to a new fate and become committed to a new lineage *via* transdifferentiation. In this project we will use as a cell model the murine cell line C2C12 derived from dystrophic mouse muscle. It is considered a mesenchymal progenitor and in cell culture can proliferate and differentiate under very specific conditions into either one of myoblast, adipocyte, chondrocyte or osteoblast phenotypes (Katagiri *et al.*, 1994; Mancini *et al.*, 2007; Ohyama *et al.*, 2002). We consider myoblast as the "default state" for the C2C12 cell since when cultured *in vitro* under growth factor limitation and without supplementation with any specific factor, it commits to myoblasts. Adding bone morphogenetic protein 2 (BMP-2), a member of the TGF-β superfamily will commit the cells to the osteoblastic lineage (Katagiri *et al.*, 1994). The role of BMP-2, TGF-β and Smads (described in detail in section 1.5.3 "The Smads and The Smads Pathway") in the acquisition of a new cell fate will be discussed in more detail in sections 1.5.1.2.1 "The Role of BMPs in Cell Commitment, Maturation and Differentiation" and 1.5.3.1.4 "Smads Function".

1.4 Cellular Signaling during Transdifferentiation *in vitro*

The study of cellular signaling processes can be done *in vivo* and *in vitro* (Furuta and Hogan, 1998; Zhang and Bradley, 1996; Gilboa *et al.*, 2000; Nohe *et al.*, 2002). While each approach has advantages and disadvantages, the detailed mechanisms of signaling pathways are better addressed *in vitro*. Studies performed in culture *in vitro* present advantages over *in vivo* models such as a better control of the experimental parameters, the ease of experimental manipulation and the ability to replicate experiments in large numbers. In contrast *in vivo* models for this study would require the generation of several different transgenic mouse lines. Also, the

chosen model system (murine cell line C2C12) offers a solid framework of dependable background information due to the available extensive literature. It is important to note that in most of the work reported on C2C12 cells, the use of conditions such as starvation, growth factor-free sera and stable and transient transfections may produce "artificial" results. Cells possess a given size pool of molecules such as ATP and when these molecules are depleted, for example due to starvation, new signaling pathways will emerge in order to maintain cell survival.

Also if a protein is overexpressed as a result of transfection, it may divert the signaling towards the production of a single protein leaving fewer resources for the expression of other proteins and therefore altering the normal cell functions. Although there are a number of articles concerning the role(s) of Smads in transdifferentiation of C2C12 cells, most, if not all experiments were based on transfection of Smads genes and therefore overexpression of Smads proteins (Yamamoto *et al.*, 1997; Afzal *et al.*, 2005; Maeda *et al.*, 2004; Zhu *et al.*, 2004; Kollias *et al.*, 2006; Lee *et al.*, 1999). Such procedures, although very informative, may subvert the intracellular signaling web. In this project it was chosen to avoid transgenic methodology while keeping in mind that findings obtained from an *in vitro* study need to be carefully interpreted and may not be directly extrapolated to *in vivo* situations.

The topic of bone origin and remodeling (the continuous replacement of bone tissue during adulthood) is important and provides great potential for research in cellular signaling and bone physiology. The importance of deciphering the mechanisms of transdifferentiation under conditions that are close to physiological may also impact on whether adult multipotent progenitor cells can substitute for embryonic stem cells in tissue repair and therefore avoid the legal/ethical considerations that influence stem cell therapy.

1.5 The TGF-β Superfamily

Intricate intercellular communication systems have evolved to ensure the proper behavior of individual cells in the context of the whole organism. Among these forms of communication, one of the most prevalent involves secreted growth factors or hormones that are recognized by membrane-bound receptors in target tissues. The activated receptors in turn, activate transcriptional regulatory factors (Massague *et al.*, 2005). To be able to differentiate, cells must respond to secreted extracellular signals through mechanisms that regulate or modulate gene expression. Between the signal and the proper elicitation of gene transcription, different cellular components are assembled as cascades or pathways to guarantee specific and successful signal transmission (Hug and Sarre, 1993).

With its 42 members in the human genome, 7 in *Drosophila melanogaster*, and 4 in *Caenorhabditis elegans*, the TGF-β superfamily forms one of the largest known families of growth and differentiation factors and is a prominent representative of this class of molecules (Massague *et al.*, 2005). The TGF-β superfamily consists of 4 groups: the TGF-βs, the activin/inhibin family, the bone morphogenetic proteins (BMPs)/decapentaplegic (Dpp)/Vg-1 family, and the Mullerian inhibiting substances (MIS) family (Roark and Greer, 1994). In species ranging from flies and worms to mammals (Shi and Massague, 2003), TGF-β super-family members exert profound effects on cell division, differentiation, migration, adhesion, organization, death (Ducy and Karsenty, 2000) and carcinogenesis (Pan *et al.*, 2005). They control the development and maintenance of most tissues (Kretzschmar *et al.*, 1997b; Nishimura *et al.*, 1998). Despite their diversity of function, all members are structurally related. They are secreted as mature peptides into the intercellular space where they form homo or heterodimers (Herpin *et al.*, 2004), and exert their effects through cellular receptors that are serine/threonine

kinases which transmit the signal from the cell exterior to the cytoplasm (Massague *et al.*, 1994). Smad proteins, phosphorylated by activated receptors can translocate to the nucleus and modulate gene expression (Attisano and Lee-Hoeflich, 2001). TGF-β signaling generally has a negative effect on cell growth, thus inactivation of this pathway contributes to tumorigenesis (Shi and Massague, 2003) and carcinogenesis where it may exert both tumor suppressor and pro-oncogenic activities depending on the stage of the tumor. Dysfunctional regulation of TGF-β has been implicated in the pathogenesis of other human diseases, including tissue fibrosis (Javelaud and Mauviel, 2004).

1.5.1 The BMP Subfamily

At the end of the 19th century it was demonstrated that decalcified bovine bone could be used for treatment of osteomyelitis. In the middle of the 20th century Lacroix raised the hypothesis of the inductive role of bone, naming the potential inducer osteogenin (Granjeiro *et al.*, 2005). The activity of BMPs was first identified in the 1960s by Urist (1965) who described them as substances that could generate the formation of ectopic bone when implanted in rat muscle. BMPs activity remained poorly studied until the purification and sequencing of bovine BMP-3 and the cloning of human BMP-2 and 4 in the 1980s (Chen *et al*, 2004). It is now evident that BMP's functions are not restricted to morphogenesis and organogenesis. These functions are not performed by all BMPs, showing the functional heterogeneity and specificity of the subfamily members (Ducy and Karsenty, 2000). BMPs, that may be active from the earliest stages of embryo development through adulthood, are important in tissue repair and tumorgenesis (Kawabata *et al.*, 1998), and are expressed all over at least in Bilateria (Herpin *et al.*, 2004). To date 20 BMP family members or ~30% of the suggested total number of TGF-β superfamily

members (Ducy and Karsenty, 2000) have been identified and characterized (Chen *et al*, 2004). For a summary see Table 1.1.

While many growth factors and hormones are implicated in bone development and regenerative processes, BMPs are important for the development and maintenance of the skeleton (Ducy and Karsenty, 2000; Wyatt *et al.*, 2001). During bone remodeling (constant replacement of bone in the adult skeleton), BMPs enhance osteoblast differentiation whereas TGF-βs stimulate osteoblast proliferation but decrease differentiation (Mundy, 2002). TGF-βs and BMPs, in particular TGF-β1 and BMP-2 are sequentially used to induce bone marrow cell differentiation *in vitro* (Jorgensen *et al.*, 2004). In the case of BMP-2 (used in this project) decisive roles during multiple steps in animal development such as dorso-ventral pattern formation, organ and tissue development and limb/wing formation have been described (Scheufler *et al.*, 1999). BMP-2 is a well-characterized inducer of mesenchymal cell differentiation (Ji *et al.*, 2000). Also, it has been found that BMP-2 is critical in chondro-osteogenic differentiation and bone development because it can stimulate both immature (bone marrow stromal cells) and differentiating osteoblasts grown from bone explants in rat models (Jorgensen *et al.*, 2004). BMP-2 has been routinely used to cause the mouse cell line C2C12 to transdifferentiate from the myoblastic to the osteoblastic lineage (Katagiri *et al.,* 1994; Rauch *et al.*, 2002; Nishimura *et al.*, 1998; Gallea *et al.*, 2001).

1.5.1.1 The Structure and Classification of BMPs

A signature of the TGF-β superfamily members is the presence of a highly conserved stretch of seven cysteine residues in their primary sequence. The BMP family can be subdivided in groups based on primary amino acid sequence homology. For example BMP-2 and BMP-4 are 92% identical in amino acid sequence while BMP-5, BMP-6, and BMP-7 are 90% identical on average.

These two groups have an approximate 60% amino acid identity (Wozney, 1992; Scheufler *et al.*, 1999). BMP-3 stands alone (Wozney, 1992). In addition BMP-1, but no otherBMP, is a metalloproteinase that can cleave chordin (an antagonist of BMP-2) and several precursors of the extracellular matrix proteins (Hartigan *et al.*, 2003). BMP-2 is the prototype of BMPs originally defined by their bone and cartilage-inductive activities at non skeletal sites *in vivo* (Scheufler *et al.*, 1999). BMP-2 is synthesized as a 453 residue pre-proprotein that is translocated into the lumen of the ER with loss of the signal peptide. The 263 amino acid proprotein becomes glycosylated probably via a galactosyltransferase and further proteolytically cleaved to yield the 114 amino acid peptide. The 114 residue protein dimerizes to yield the mature homo or hetero dimeric protein. Each monomer is a single domain protein (Scheufler *et al.*, 1999). BMP-2 is stabilized by hydrophobic interactions, which are further strengthened by an inter-subunit disulfide bridge (Shi and Massague, 2003).

The BMP-2 molecule has two epitopes (amino acid sequence of a given protein with characteristics that are likely to induce interaction with other molecules) that interact with the appropriate receptors in order to initiate the cellular signal (Figure 1.2).

1.5.1.2 The Functions of BMPs

BMPs are multifunctional proteins with pivotal roles in embryonic pattern formation (morphogenesis and organogenesis), regulation of differentiation, proliferation, migration and survival of many cell types (Ducy and Karsenty, 2000; ten Dijke, 2006), adult tissue homeostasis (Knaus and Sebald, 2001) and, in vertebrates, the formation of cartilage, bone and the supporting tissues of the skeleton (Wozney and Rosen, 1998). However, BMPs have effects unrelated to bone formation (Yamashita *et al.*, 1995) and thus are expressed in many soft tissues (Hogan, 1996) in addition to skeletal tissues (Table 1.1).

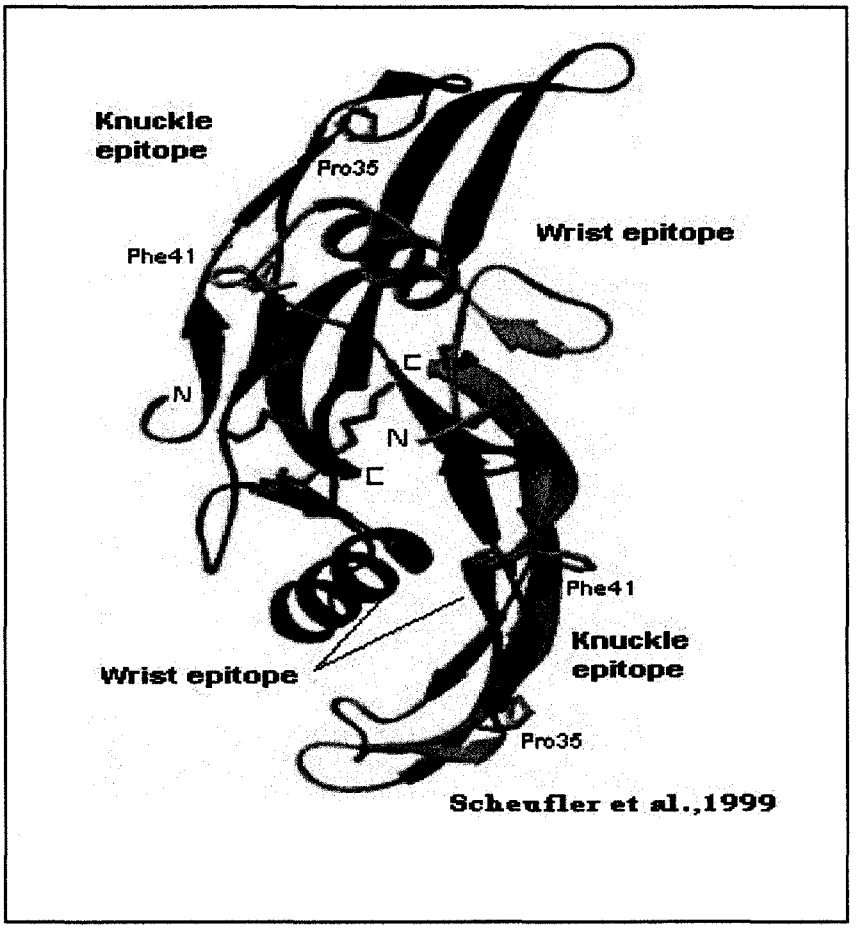


Figure 1.2 Folding topology of the native BMP-2 dimer. α-helices are indicated as spirals, β-strands as arrows, disulfide bridges are shown as green sticks. The subunits are color-coded blue and orange, respectively. The interactions (Phe41, Pro35) that are responsible for dimer formation between a helix and the β-strands of the other subunit are visible. N and C represent the protein terminals.

BMP	Tissue(s) of high expression	Alternative names	Phenotype
BMP-2	Cartilage, bone	BMP-2a	Lethal
BMP-3	Cartilage, bone, ovary	Osteogenin	
BMP-3b	Muscle, bone, brain, lung	GDF-10	
BMP-4	Cartilage, bone, prostate	BMP-2b	Lethal
BMP-5	Bone		Viable, with skeletal defects
BMP-6	Cartilage	Vgr-1	
BMP-7	Bone, kidney	OP-1	Lethal
BMP-8a,b	Testis, placenta	OP-2, OP-3	
BMP-9	Liver	GDF-2	
BMP-10	Bone, heart		
BMP-11	Olfactory neurons progenitors *	GDF-11	
BMP-12	Cartilage	GDF-7, CDMB-3	
BMP-13	Cartilage	GDF-6, CDMB-2	
BMP-14	Cartilage	GDF-5, CDMB-1	
BMP-15	Oocytes	GDF-9	

Table 1.1 Bone morphogenetic protein family. The BMP family genes are expressed in numerous tissues and often are quoted with other names. Available on line at http://herkules.oulu.fi/isbn9514266064/html/b376.html. * Not a tissue, but a cell.

A study comparing the osteogenic activity of fourteen human BMPs was conducted by expressing the BMPs in two mouse cell lines (pluripotent, mesenchymal precursor CH310T1/2 and pre-myoblastic C2C12) and the human committed osteoblastic cell line TE-85. The 14 BMPs were transiently transfected into the cells and expression of the different BMPs assessed. The osteoblastic lineage-specific commitment/differentiation of these cell lines was monitored by measuring alkaline phosphatase (ALP) activity over time. In the less differentiated CH310T1/2 cell line, only BMPs 2, 6, and 9 elicited ALP expression with BMP6 being the most potent. Expression was highest at day 9. In C2C12 cells, BMPs 2, 4, 6, 7 and 9 elicited ALP expression with BMPs 2 and 9 inducing the highest levels of ALP at 5 days in culture. TE85 cells are committed to osteoblasts and relatively low expression of ALP was induced by all the BMPs (Cheng *et al.*, 2003). BMP-9 also acted as a potent synergistic factor for hematopoietic progenitor-cell generation and colony formation (Ploemacher *et al.*, 1999). Comparisons of activity among the BMPs in Cheng's paper were based on intracellular not extracellular expression of BMPs as would be the case *in vivo*.

The results of experiments performed *in vivo* are consistent with *in vitro* data. Studies on the gain or loss of function of BMPs in mice demonstrate that they play critical roles in cartilage and bone formation as well as being important during mouse development, the function of cardiovascular, pulmonary, reproductive and urogenital organs in adults and in the nervous system (Goumans and Mummery, 2000). Homozygous mutant BMP-4 embryos die between 6.5 and 9.5 days post coitum (dpc) from varying defective phenotypes (Winnier *et al.*, 1995). Homozygous BMP-7 deficient mice die shortly after birth because of poor kidney development (Luo *et al.*, 1995) or present kidney and eye development problems at birth (Dudley *et al.*, 1995). Deletion of BMP-2 in transgenic mice caused embryonic lethality when homozygous. Mutant embryos failed to close the proamniotic canal (structure surrounded by cells

that appears early after fertilization), which caused malformation of the chorion (the outermost of the two fetal membranes that enclose the embryo) and amnion (fetal membrane of reptiles, birds, and mammals that lines the chorion). BMP-2-deficient embryos also exhibited a defect in cardiac development. These results have lead scientists to attempt to identify the best osteogenic BMPs. *In vivo* experiments have confirmed that all the BMPs do not have the same potential to induce the formation of bone in adult tissue (O'Keefe, 2005; Hunt *et al.*, 1996) and preclinical trials have identified and led to the clinical introduction of the most potent BMPs (BMP-2 and BMP-7) *in vivo* (Ripamonti *et al.*, 2001; Einhorn, 2003; Termaat *et al*, 2005).

1.5.1.2.1 The Role of BMPs in Cell Commitment, Maturation and Differentiation

BMPs function as inducers of lineage commitment in multiple embryonic developmental processes including hematopoiesis. Also during gastrulation they are responsible for dorsoventral patterning within the neural tube, and the induction of mesoderm. (Attisano and Wrana, 2002; Varga and Wrana, 2005). The expression of BMPs in the embryo occurs very early, e.g., expression can be found as early as 8.5 dpc for BMP-2, 6.5 dpc for BMP-4, 10.5 dpc for BMP-5 and 8.5 dpc for BMPs 6 and 7 (Ducy and Karsenty 2000). As might be expected from their complex *in vivo* functions BMPs play key roles in regulating fate choices during stem cell differentiation (Varga and Wrana, 2005), and in neural crest stem cell (NCSCs) lineage acquisition (Shah *et al.*, 1996). The critical contribution of BMPs is to induce the expression of inhibitor of DNA binding (Id) genes via the Smads pathway (Ying *et al.*, 2003). In general, Id proteins favor proliferation and are dominant negative regulators of differentiation through the inhibition of basic helix-loop-helix (bHLH) proteins such as MyoD that favor expression of lineage specific genes during differentiation-commitment (Atherton *et al.*, 1996). Studies on the

roles of BMPs in regulating embryonic stem cells (ES) compared with NCSCs strongly suggest that combinations of extracellular factors regulate stem cells, and that crosstalk between intracellular signaling pathways precisely define stem cell fate commitment (Varga and Wrana, 2005).

Cell commitment *per se* seems to be affected by different factors. Numerous studies *in vitro* have evaluated the individual functions of BMPs in different cell lines representative of multipotent mesenchymal cells, osteoblastic progenitor cells, osteoblasts, chondroblasts and osteosarcoma cells (Sakou, 1998). In the case of mesenchymal precursors, BMP-2 treatment of the murine cell line C2C12 and rat cell line ROB-C26 (capable of transdifferentiatiation to either myoblasts, osteoblast or adipocytes), induced the expression of ALP in C2C12 and ALP and osteocalcin in ROB-C26, signatures of commitment towards the osteoblastic lineage (Katagiri *et al.*, 1994; Suda *et al.*, 1999; Takagi *et al.*, 2004). Whether BMP is going to induce or inhibit proliferation is influenced by the cell type, its differentiation stage and the culture conditions (Sakou, 1998). Some BMPs function more effectively in committed osteoblasts and others are more effective in less differentiated cells (Centrella *et al*, 1998).

1.5.1.2.2 BMPs in Transdifferentiation

Treatment of mouse pluripotential mesenchymal precursor C2C12 cells with BMP-2 when cultured in FBS limited medium can cause transdifferentiation from the myoblastic to the osteoblastic lineage (Figure 1.1). In overview, BMP-2 causes the repression of genes involved in the acquisition of the myoblastic fate while simultaneously enhancing the expression level of genes associated with the osteoblastic fate (Katagiri *et al.*, 1997; Lee *et al.*, 1999; Murray *et al.*, 1993).

A number of authors have studied the cause(s) of this shift in lineage and as a result many different proteins and pathways have been linked to the differentiation process. Yang *et al.*, (2000), studying the induction of osteoblastic transdifferentiation in C2C12, reported that the level of mesenchyme forkhead-1 (MFH-1) mRNA and protein increased after treatment with BMP-2. This suggests that MFH-1 may play a key role in regulating this commitment and the production of osteoblast markers. Winnier *et al.*, (1997) have shown that MHF-1 is required in the development of the paraxial mesoderm and related structures. Rawadi *et al.*, (2003), suggested that the capacity of BMP-2 to induce ALP relies on wingless/Int (WNT) expression and the lipoprotein-receptor-related protein *5* (LRP5) signaling cascade. Nishimura *et al.*, (1998), suggested that the activation of Smad-5 and subsequent formation of the Smad-5/Smad4 complex, translocation to the nucleus and activation of genes by these proteins is key to the transdifferentiation process. Since the signal that started with the BMPs affected the transcription of a high number of genes, variation in the expression level of many genes and proteins is expected.

The signal that drives the transdifferentiation from myoblastic to osteoblastic as well as many other cellular responses via transcriptional changes starts with the binding of BMPs to specific receptors that are present in the cellular membrane and will pass the signal to the interior of the cell. The next section focuses on the properties of these receptors.

1.5.2 The BMP Receptors I and II

1.5.2.1 General Characteristics

The BMP receptors belong to the TGF-β receptor family. Their fundamental role is to transmit the signal initiated by the ligand. Due to their similarity, the TGF-β and BMP pathways have been assumed to work in a comparable manner, with TGF-β as the prototype. Based on their

structural and functional properties, the TGF-β and BMP receptor families are divided into two subfamilies: type I and type II (Herpin *et al.*, 2004; Gilboa *et al.*, 2000) see Fig 1.3 and Table 1.2. Both subfamilies are transmembrane serine/threonine kinases (Gilboa *et al.*, 2000; ten Dijke *et al.*, 2004) distinguishable by their protein structure. In mammals, five type II receptors and seven type I receptors (the type I also called activin receptor-like kinases or ALKs) have been recognized (Shi and Massague, 2003; Bertolino *et al.*, 2005) (Fig 1.3). In mammals, BMPs bind to ALK-2, ALK-3 and ALK-6 whereas TGF-βs bind to ALK-5 and ALK-1 and activins bind ALK-4 (Maeda *et al.*, 2004).

As a result of being simultaneously cloned, most type I receptors have received multiple names. One practice has been to use the neutral nomenclature ALK until the ligand becomes identified. Of interest in this project are BMP receptor type I (BMPR-IA and -IB) previously known as ALK3 and ALK6, respectively. ALK2 known as Activin receptor type I (ActR-I) binds activin and BMP-2 and 4 (Massague, 1998). In vertebrates, the type II receptor subfamily includes TGF-β type II (TRß-II), BMP type II (BMPR-II) and anti-Mullerian hormone receptor (AMHR) which selectively bind TGF-β, BMPs and MIS respectively. ActR-II and ActR-IIB bind activins when expressed alone or in concert with activin type I receptors. However, ActR-II and ActR-IIB can bind BMPs 2, 4, and 7 and growth and differentiation factor 5 (GDF5) in concert with BMP type I receptors (Massague, 1998). At the protein level, type I TGF-β receptors have sizes of ~53 KDa and type II receptors of ~73 KDa (Centrella *et al.*, 1995). These receptors have three domains: an extracellular domain, a single membrane spanning domain and a kinase intracellular domain containing the serine/threonine region (Yamashita *et al.*, 1996). The characteristic serine-glycine rich sequence or GS domain (SGSGSG) preceding the kinase domain in the type I receptor distinguishes the two receptors (Fig 1.4). Phosphorylation of the GS

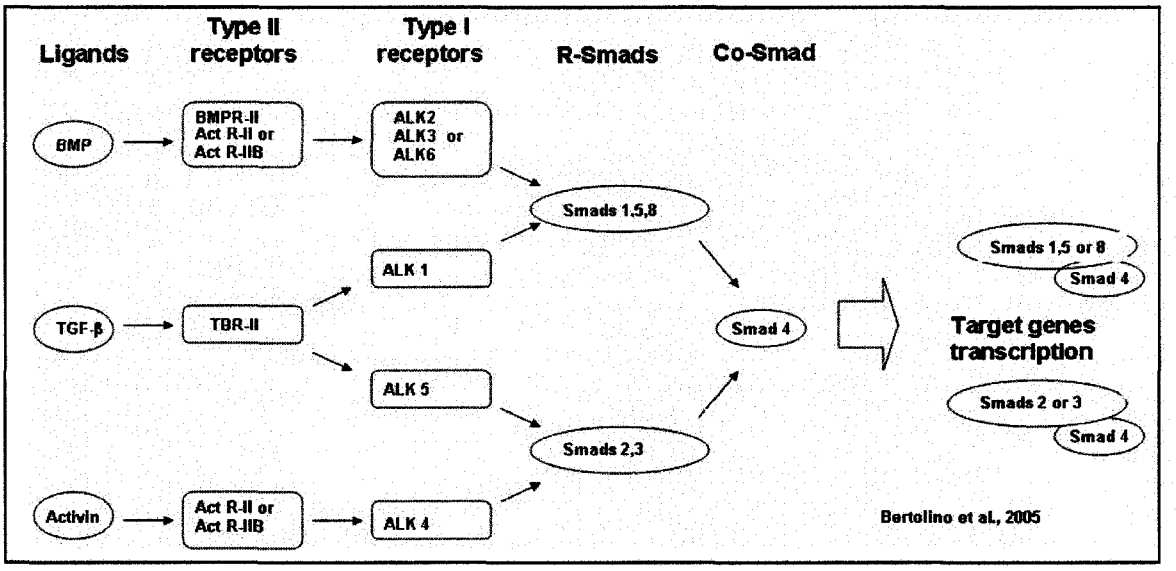


Figure 1.3 The TGF-β superfamily of receptors and their substrates. Ligands act through specific receptors sometimes common to TGF-ßs and BMPs, to induce signaling through selective pathways (here the Smads pathways). Divergence and convergence in TGF-ß signaling through receptors and Smads is observed. ALK 7 (not depicted in the figure), is the receptor for Nodal a component of the TGF-ß superfamily that also phosphorylates Smads 2/3 (Bertolino et al., 2005). AHMR, a type II receptor not depicted in the picture binds MIS.

Receptors Type I	Other Name Used	Receptors Type II	Other Names Used
BMPR-IA	ALK-3, BRK-I, TFR-11*	ActR-II	
BMPR-IB	ALK-6, BRK-2*	ActR-II B	
ActR-I	ALK-2, R1, Tsk-7L*	BMPR-II	BRK-3, T-ALK*
ActR-IB	ALK-4		

Table 1.2. Type I and II receptors involved in BMP signaling. BMP signaling receptors type I and type II and other names assigned to them. *From Yamashita, 1996

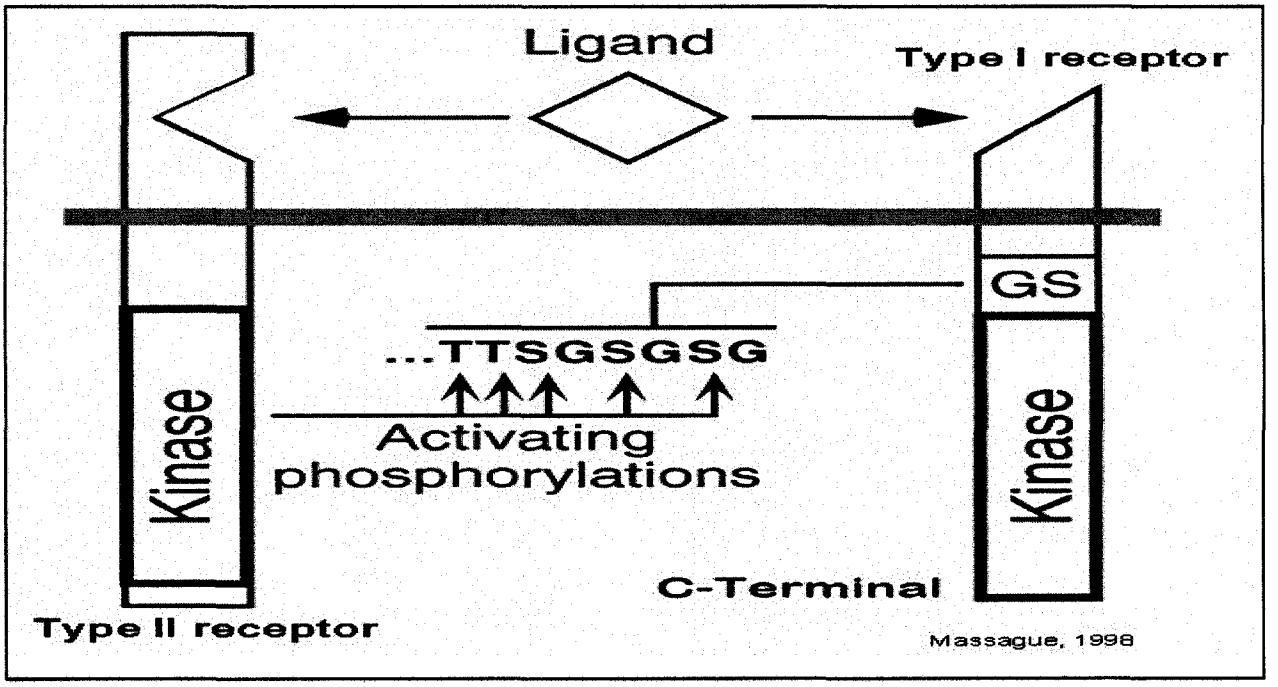


Figure 1.4 Type I and II TGF-β receptor phosphorylation. In type I receptors, the protein kinase domain is preceded by the GS domain (GS). The characteristic GS sequence motif of TβR-I is shown, indicating the potential phosphorylation sites.

domain by BMPR-II during signal transduction may regulate the catalytic activity of the type I receptor or its interaction with substrates (Massague 1998). Signaling activity does not depend on any particular serine in the GS domain, but rather on the number of unphosphorylated residues (Franzen *et al.*, 1995). Mutation of two or more of five potential consensus sites (TTSGSGSG) (Fig 1.4) in the middle of the GS domain impairs phosphorylation and signaling activity (Wieser *et al.*, 1995).

The cytoplasmic C-terminus of BMPR-II functions as a docking site for many regulatory proteins, several of which have been analyzed. The most represented group comprises regulatory proteins that provide links to signaling pathways involved in the regulation of multiple cellular functions initiated through Smad-independent signaling (Hassel *et al.*, 2004).

1.5.2.2 Receptor-Receptor and Ligand-Receptors Interactions

Specific amino acid sequences mediate interactions in the BMP pathway including interactions between type I and II receptors and between receptors and ligands. Two non-overlapping regions or receptor binding epitopes, the "wrist" and "knuckle" (Fig 1.2) determine the high binding affinity of BMP-2 for either BMPR-IA or BMPR-1B or the low binding affinity of BMP-2 for BMPR-II respectively. Further analysis might be needed to more clearly define the contacts between the BMP-2 molecule and the receptors (Kirsch *et al.*, 2000; Nickel *et al.*, 2001).

Type I and II TGF-β and BMP receptors oligomerize in different combinations that may elicit alternative signal amplification downstream of the formed complexes (Nohe *et al.*, 2002). Several models have been proposed to explain the two-step assembly of a functional signaling complex for TGF-βs. In the allosteric model, the binding of ligand to the type II receptor is required to induce a conformational change in the ligand, which leads to exposure of the binding epitope for the type I receptor. In the cooperative model, the ectodomain (extracellular part of the

receptor) of the type I receptor interacts with an extended surface that relies on the formation of the type II receptor-ligand complex. In this model, the ectodomains of the type I and type II receptors do not directly contact each other but bind cooperatively to the ligand (Shi and Massague, 2003). Gilboa *et al.*, (2000) in a study aimed at deciphering the differences of complex oligomerization between TGF-β and BMP receptors, demonstrated that the oligomerization pattern of the BMP receptors differs from that of the TGF-β receptors being more flexible and more susceptible to modulation by ligands. BMP-2 was found to increase significantly the homo-oligomerization of BMPR-IA and -IB, thus raising the possibility that homomeric interaction within these complexes may be unique and serves to regulate functional responses. It was also demonstrated that type I/type II receptors have an intrinsic affinity for each other and that this affinity is increased after the binding of BMP-2. BMP-2 binds very effectively to BMPR-IA and -IB and very weakly to BMPR-II unless the latter is interacting with a type I BMP receptor. According to Hassel *et al.*, (2004), BMP-2 binds with high affinity to the bone morphogenetic receptor type I (BMPR-I) or to a preformed heteromeric complex. Nohe *et al.*, (2002) confirmed the flexibility of oligomerization of BMP receptors and gave the ligand, in this case BMP-2, two options to initiate signal transduction: either binding to preformed receptor complexes activating them or binding to the high affinity type I receptors that subsequently recruit unliganded type II receptors into the ligand-mediated signaling complex. After ligand binding and receptor activation, the type II receptor activates the type I receptor via phosphorylation enabling it to phosphorylate the Smads or p38 proteins thus activating their respective pathways (Fig 1.5).

1.5.3 The Smads and the Smads Pathway

Smads are a group of proteins widely expressed throughout embryonic development and in most adult tissues and cell types. They are critical for transmitting signals started by the TGF-β

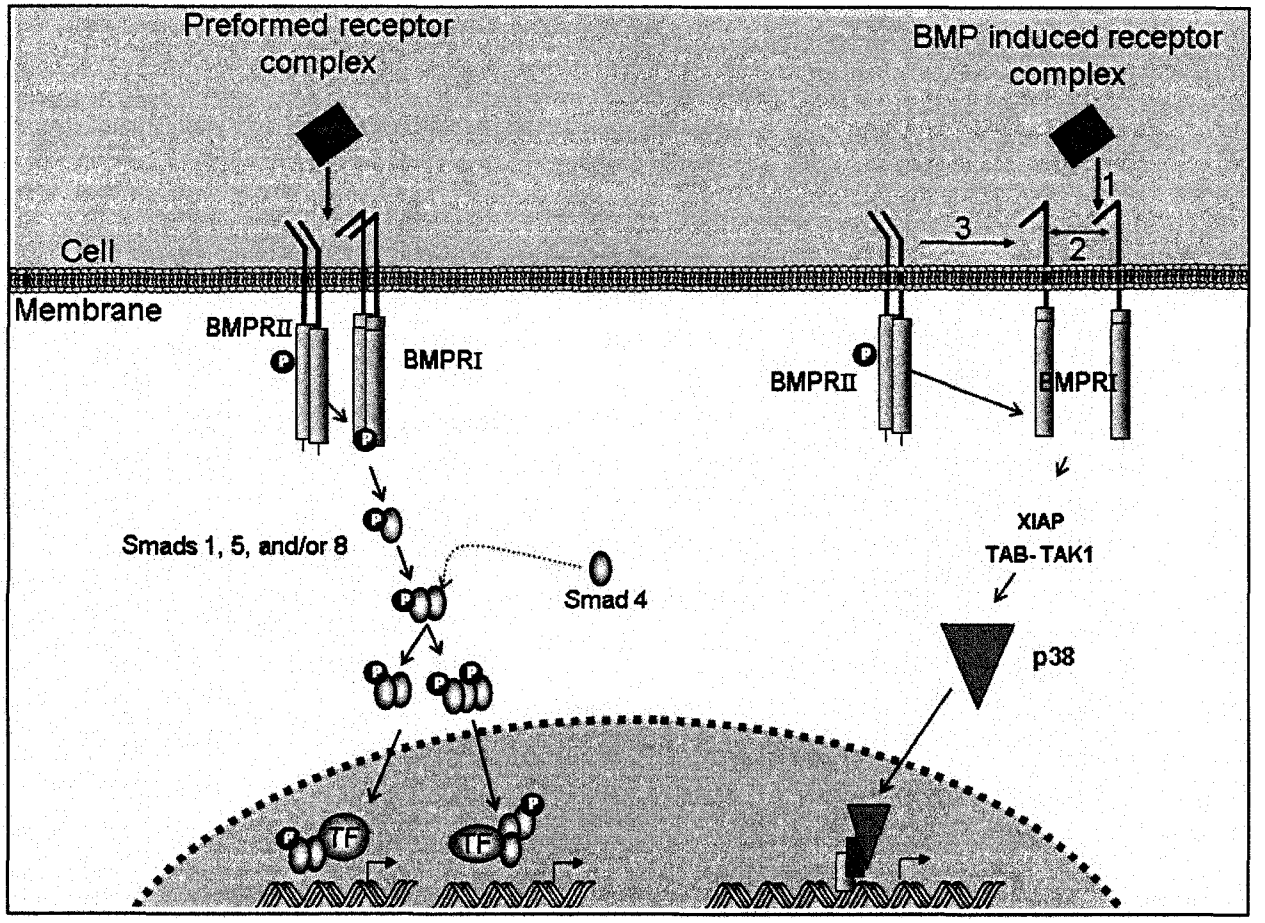


Figure 1.5 Preformed and BMP induced receptors. The induction of the Smads and p38 pathways depends on the receptor that is liganded by BMP-2. Preformed receptor complexes elicit the Smads pathway whereas BMP-2 induced receptor complexes elicit the p38 pathway. The numbers indicate the stepwise formation of the BMP-2 induced receptor complex. The Smads and p38 pathways are diagrammatically described.

superfamily from the cell surface to the nucleus, (Attisano and Lee-Hoeflich, 2001).

The name "Smad" was coined with the identification of human Smad-1 in reference to its relatedness to the gene *sma* in C. *elegans* and "mother against decapentaplegic" (MAD) in *Drosophila* (Liu *et al.*, 1996). Typically, the Smads activated (phosphorylated) by TGF-βs are Smads 2 and 3. Smads 1, 5 and 8 are induced by the BMPs. Once phosphorylated, the Smads bind Smad-4 to be translocated into the nucleus.

1.5.3.1 Smads Classification Structure and Function

Eight mammalian members of the Smad family have been reported (Kawabata *et al.*, 1998; Attisano and Lee-Hoeflich, 2001; Shi and Massague, 2003; Miyazono *et al.*, 2005; Massague, *et al.*, 2005) with sizes ranging from about 400 to 500 amino acids in length (Attisano and Lee-Hoeflich 2001). They are divided into different classes:

- Smads that participate in positive signaling include the receptor regulated Smads (R-Smads 1, 5, 8, 2, 3) and the common mediator Smad-4 (Co-Smad) the latter is shared by both BMP and TGF-β/activin signaling pathways (Miyazono *et al.*, 2002, 2005).
- The interference or inhibitory Smads (I-Smads 6 and 7) that negatively regulate signaling mainly through inhibition of the R-Smads and the Co-Smad (ten Dijke and Hill, 2004).

R-Smads 1, 5 and 8 are phosphorylated by BMPR-I receptors whereas R-Smads 2 and 3 are phosphorylated by ActR-I and TRβ- I (Miyazono *et al.*, 2005). Once phosphorylated, R-Smads bind to Smad-4 forming complexes that head to the nucleus leading to an accumulation of Smads that bind specific transcription factors and regulate the expression of many genes including those that encode other transcription factors. *Ca.* 500 genes are regulated this way. Typically two-thirds are activated by the signal and the rest are repressed (Massague *et al.*, 2005).

1.5.3.1.1 The MH1 Domain

Smads possess highly conserved N- and C-terminal regions with homology to MAD proteins (MH; hence MH1 and MH2 domains) that mediate many Smads functions (Itoh *et al*, 2000). These domains are separated by a region that includes a proline-rich linker region of varying length (Attisano and Lee-Hoeflich, 2001; Miyazono *et al.*, 2005) (Figure 1.6).

MH1 domains are conserved in R-Smads and the Co-Smad, but not in I-Smads (Miyazono *et al.*, 2005; Massague *et al.*, 2005). The MH1 domain of the R-Smads has a DNA binding function and a MH2 domain repression function (Miyazono *et al.*, 2005). The crystal structure of the MH1 domain of Smad-3 bound to an 8 base-pair Smad-binding element (GTCTGTCT) demonstrates that the MH1 domain forms a compact globular fold that uses a highly conserved 11-residue β hairpin to contact DNA in the major groove (Fig 1.7). This hairpin structure is conserved in all the R-Smads and Smad-4 (Massague *et al.*, 2005). In Smad-2, a 30 amino-acid insertion encoded by exon 3 is thought to displace the β-hairpin loop, providing a structural explanation for Smad-2's lack of DNA-binding activity. The interaction of Smads with several transcription factors, including Jun, TFE3, Sp1 and Runx, also occurs through the MH1 domain. (Attisano and Lee-Hoeflich, 2001).

1.5.3.1.2 The MH2 Domain

MH2 domains are highly conserved in all Smad proteins (Miyazono *et al.*, 2005; Massague *et al.*, 2005). The first resolved crystal structure of a Smad MH2 domain was that of Smad-4 (Attisano and Lee-Hoeflich, 2001). This study revealed that the MH2 domain is composed of five α helices (H1 to H5) and three loops (L1, L2 and L3) that enclose a β sandwich. The MH2 domain does not bind DNA and is instead a multifunctional region that mediates differential association with a wide variety of proteins (Shi and Massague, 2003). MH2 domains play important roles in

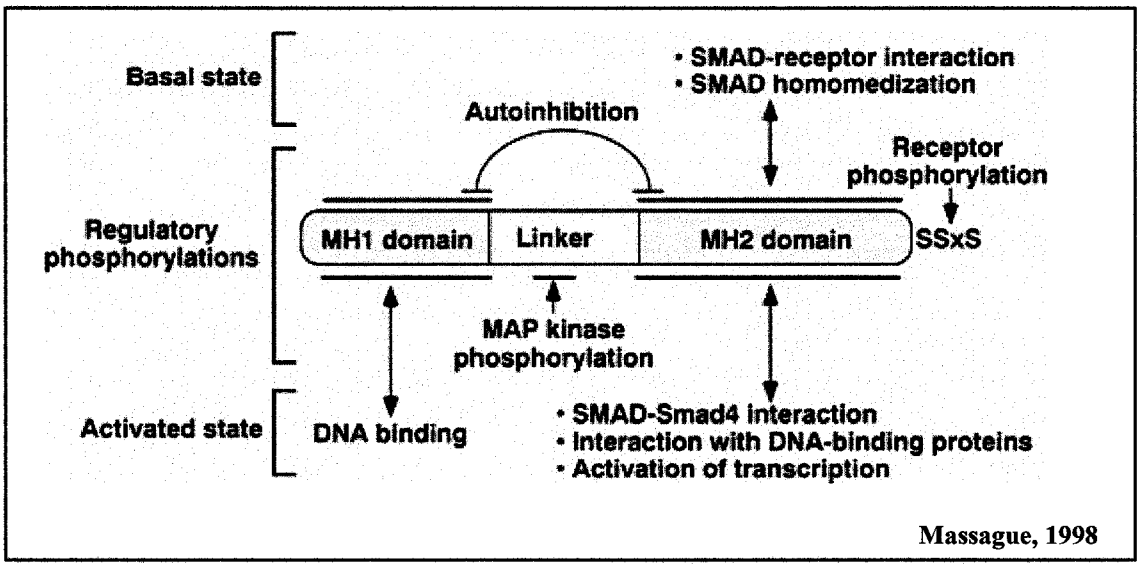


Figure 1.6 Smad domains and their functions. Representation of the Smads structural fundamentals. The MH1 and MH2 domains of the Smads proteins have different functions depending on their state.

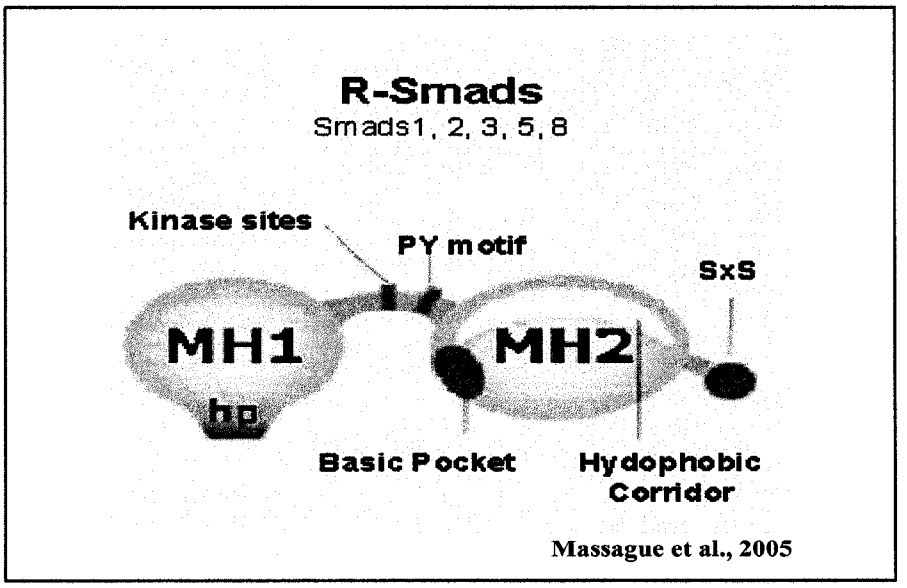


Figure 1.7 Smad proteins and their structural elements. The MHI domain contains the hairpin (hp) structure for DNA binding. The PY motif is recognized by the Smurf ubiquitin ligases. The linker region of R-Smads has sites that are phosphorylated by kinases. The basic pocket interacts with type I receptors. The hydrophobic corridor mediates interactions with proteins such as retention proteins, nucleoporins, and with DNA-binding cofactors (Massague et al., 2005)

interaction with type I receptors, oligomer formation with other Smads, and transcriptional activation (Miyazono *et al.*, 2005). A set of contiguous hydrophobic patches, referred to as the "hydrophobic corridor", on the surface of MH2 mediates interactions with cytoplasmic retention proteins, components of the nuclear pore complex (nucleoporins) and with DNA-binding cofactors (Massague *et al.*, 2005) (Fig 1.7). The MH2 domain mediates direct contact with the nuclear pore complex for nucleocytoplasmic shuttling (Shi and Massague, 2003). Smads exist as monomers and trimers, and the MH2 domain is responsible for formation of homomeric as well as heteromeric Smad complexes (Attisano and Lee-Hoeflich, 2001; Shi and Massague, 2003).

Analysis of the crystal structure of trimeric Smad-4 demonstrated that the loop-helix region (L1, L2, L3 and H1) of one subunit makes extensive contacts with the three-helix bundle (H3, H4 and H5) of another subunit and that many conserved residues in Smads are located within the trimer interface. The overall topology of the R-Smad MH2 domain is similar to that of Smad-4, but in addition, the R-Smads have a basic pocket on one surface that lies adjacent to loop 3 (Wu *et al.*, 2000). As Smad-4 does not interact with receptors, it is thought that this basic pocket may serve as a docking site for the phosphorylated and activated type I receptors (Attisano and Lee-Hoeflich, 2001). Smad-4 shares most of its carboxyl terminal sequence with R-Smads but lacks the serine rich motif (SSXS or SSVS) at the C-terminal end. Since this motif is phosphorylated by type I receptors upon ligand stimulation (Miyazono *et al.*, 2005; Massague *et al.*, 2005), there is no phosphorylation of Smad-4 (Kerzschmar *et al.*, 1997a).

1.5.3.1.3 The Linker Region

The linker region that connects the MH1 and MH2 domains (Fig 1.6) contains a number of important peptide motifs including:

- potential sites for phosphorylation by mitogen-activated protein kinases (MAPKs) that may regulate R-Smad function;
- nuclear export signal (NES) in Smad-4 for recycling to the cytoplasm (Massague *et al.*, 2005);
- conserved proline-tyrosine (PY) motif in R-Smads and I-Smads that mediates interaction with the WW domains (small modular binding domains that recognize polyproline motifs, Chong *et al*, 2006) of the Smad ubiquitin regulatory factors Smurf1 and Smurf2 involved in protein degradation (Massague *et al.*, 2005; Attisano and Lee-Hoeflich, 2001) (Fig 1.7);
- Smad activation domain (SAD) in Smad-4 required for transcriptional activation (Attisano and Lee-Hoeflich, 2001).

There is experimental evidence for several of these functions. In the case of the linker region of Smad-1, four PXSP motifs (consensus sites for MAPKs) are located at serines 187, 195, 206, and 214. Using directed mutagenesis researchers found that PXSP motifs in the linker region of Smad-1 are phosphorylated independently of the C-terminal motifs, and that their phosphorylation is mediated by a kinase other than the BMP receptor. In this case, Smad-1 is a target of the ERK subfamily of MAP kinases. It is suggested that ERK-mediated phosphorylation of the linker region inhibits nuclear accumulation of Smad-1 and is capable of preventing BMP-induced nuclear accumulation of Smad-1 without interfering with formation of the Smad-1/Smad-4 complex (Kretzschmar *et al.*, 1997b). Crystal structure analysis of a fragment of Smad-4 that included the SAD and MH2 domain revealed that SAD contacts a Smad-4-specific sequence in the MH2 domain that stabilizes a glutamine-rich α-helical extension termed the TOWER, which, together with the proline-rich SAD, may form a transcriptional activation surface (Attisano and Lee-Hoeflich, 2001).

1.5.3.1.4 Smads Function

Smads lie at the core of the BMP and TGF-β signaling pathways that control activities such as cell division, differentiation, migration, adhesion, organization and death. These functions are accomplished via the R-Smad/Co-Smad complexes formed in the cytoplasm that then translocate to the nucleus where they integrate DNA-binding cofactors such as transcriptional coactivators or co-repressors that give the complex selectivity. The complexes bind DNA and elicit the selective expression of defined genes (Massague *et al*, 2005). Individually, R-Smads function as substrates for the BMPR-I. They bind to the Co-Smad and have a motif in the linker region that allows for their nuclear accumulation. The Co-Smad basically joins either R-Smads to form the translocating complex or I-Smads forming complexes that contribute to signaling control (Massague, 1998).

I-Smads in the cytoplasm compete with Co-Smad for R-Smads to impede Co-Smad/R-Smads oligomer formation, thus controlling the R-Smads effects on transcription (Miyazono, 2002). They also negatively regulate TGF-β signaling by competing with R-Smads for receptor interaction and by targeting receptors for degradation (Shi and Massague, 2003). A puzzling characteristic of I-Smads is that in some cells, they are detected in the nucleus but not in the cytoplasm (Miyazono, 2002). Inhibitory Smads participate in negative feedback loops that may regulate the intensity or duration of BMP-2 responses. For example, at low levels, Smad-6 does not interfere with receptor-mediated phosphorylation of Smad-1 but competes with Smad-4 for binding to activated Smad-1, appearing to act as a Smad-4 decoy for BMP-activated Smads (Massague, 1998). Smad-6 also inhibits the phosphorylation of Smad-1 at higher concentration (Imamura *et al.*, 1997). Smad-6 mainly inhibits TGF-β signaling by associating with type I receptors, interfering with the phosphorylation of Smad-2 and the heterodimerization with Smad-4. Smad-6 acts as well at the nuclear level, for example by repressing the BMP induced

transcription of Id1 (inhibitor of DNA binding 1) proteins involved in cell proliferation and differentiation. Repression works by the recruitment of the transcriptional co-repressor C-terminal binding protein (CtBP) (Lin *et al.*, 2003). Smad-7 resides in the nucleus, and ligand stimulation results in its export into the cytoplasm where it can bind to receptors, thus exerting its inhibitory effects (Attisano and Lee-Hoeflich, 2001). It inhibits TGF-β/activin and BMP signaling by binding to the activated receptors and hence competing with R-Smads (Nakao *et al.*, 1997). Smad-7 has been shown to play an important role in regulating TGF receptor ubiquitination by Smurf ubiquitin ligases. The Smurf–Smad-7 complex, after exiting the nucleus, binds to the TGF receptor complex and promotes its ubiquitination, thereby favoring down-regulation. BMP receptors are also targeted for degradation in this manner, and it appears that Smurf1, as well as Smads 6 and 7, plays a role during this process (Massague *et al.*, 2005).

1.5.3.1.5 Smads Defects and Diseases

The analysis of mouse embryo mutants has revealed variation in the pattern, timing and level of expression of the individual Smads (Attisano and Lee-Hoeflich, 2001). To date, five of the eight Smad genes have been disrupted by homologous recombination in mice. Mice lacking Smad-2 (Weinstein *et al.*, 1998; Heyer *et al.*, 1999), Smad-4 (Sirard *et al.*, 1998; Yang *et al.*, 1998.), or Smad-5 (Yang *et al.*, 1999; Chang *et al.*, 1999) all have an early embryonic lethal phenotype (Table 1.3). In the case of Smad-2 (Waldrip *et al*, 1998; Nomura and Li, 1998) and Smad-4 (Yang *et al.*, 1998) null mutant mice have defects in mesoderm induction and anterior-posterior axis formation, whereas Smad-5 null mice die later, between embryonic days 9 and 11.5, and display defects in angiogenesis (Yang *et al.*, 1999; Chang *et al.*, 1999). It is interesting to note that mice heterozygous for Smad-2 (Nomura and Li, 1998), Smad-3 (Zhu *et al.*, 1998) or Smad-4 (Takaku *et al.*, 1999) also display various defects, indicating that Smad gene dosage is

Knockout	Viability	Phenotype
Smad-2	Embryonic lethal	Defects in mesoderm induction, anterior-posterior and left-right patterning, extra-embryonic tissues and endoderm formation (before E8.5)
Smad-3	Viable	Variable phenotypes including defects in T-cell and splenocyte responsiveness; metastatic colon cancer, accelerated wound healing, and degenerative joint disease
Smad-4	Embryonic lethal (E6.5-8.5)	Defects in gastrulation and anterior development, epiblast Proliferation and egg cylinder formation. Heterozygotes have intestinal tumors
Smad-5	Embryonic lethal (E9.5-11.5)	Defects in angiogenesis, vasculogenesis, left-right axis determination, and primordial germ cell development
Smad-6	Viable	Multiple cardiovascular abnormalities: defects in cardiac valves and outflow tract septation; aortic ossification, and elevated blood pressure

Table 1.3. Phenotypes of Smad deficient mice. Phenotypes of mice after knockout of selected Smad genes.

important. Several groups have independently targeted the Smad-3 gene, and each report distinct phenotypes. Those include defects in T-cell or splenocyte responsiveness (Datto *et al.*, 1999; Yang *et al.*, 1999b); development of colorectal cancers (Zhu *et al*, 1998) and degenerative joint disease resembling osteoarthritis (Yang *et al.*, 2001). Smad-3-deficient mice can be viable and fertile (Zhu *et al*, 1998). Mice lacking the inhibitory Smad-6 have cardiovascular abnormalities, including hyperplasia of the cardiac valves and outflow tract septation defects, suggesting that Smad-7 cannot substitute for Smad-6 even though both are highly expressed in the cardiovascular system (Galvin *et al.*, 2000).

Mutations in Smads have been implicated in a number of human cancers. Nuclear Smad-8 was present in normal/benign prostate tissue but absent in higher grade prostate cancerous cells and adjacent hyperplasia (Horvath *et al.*, 2004). The loss of Smad-8 expression is associated with multiple types of cancers, including 31% of both breast and colon cancers (Cheng *et al.*, 2004). Smad-4 was originally identified as a potential tumor-suppressor gene in pancreatic carcinoma; and mutations in colorectal, and pancreatic tumors (Miyazono, 2002). Smad-4 is also mutated in families with familial juvenile polyposis, an inherited syndrome associated with an increased risk of gastrointestinal cancer. Consistent with these observations, Smad-4 heterozygote mice develop intestinal polyposis (Takaku *et al.*, 1999) and invasive carcinomas. Smad-2 has also been shown to harbor mutations in colorectal (Xie *et al.*, 2003) and lung tumors (Riggins *et al*, 1997).

1.5.3.1.5.1 Smads and Osteogenesis-related Disorders

There are several medical applications to which a detailed knowledge of the signaling pathways leading to the osteoblastic lineage could contribute; the results of this project may be relevant to Smads and bone-related disorders. Disruption of Smads pathways in mice has produced embryonic lethality, cancer, degeneration and other disorders (Attisano and Lee-

Hoeflich, 2001; Itoh *et al*, 2000). In human, loss of bone mass occurring with age may benefit from transfer of satellite cells to regenerate the lost bone. Also, the fact that bone cancer is a growing concern (the third most common location where cancer cells metastasize is bone, Roodman, 2004) has prompted researchers to investigate the role of Smads in this disease. Yearly, mutations in Smads genes are the cause of about 140,000 new diagnosed cancers in the USA (Takaesu *et al.*, 2005). Furthermore the loss of function of Smads has been related to pathologies such as pancreatic and colorectal cancers (Miyazono, 2002), and a significant proportion of breast, ovarian, prostatic, esophageal and gastric cancers as well (Massague, 1998). More specifically, mutations in Smads 2 and 4 are detected in 50% of pancreatic cancers, 20% of colon cancers and 10% of lung cancers (Riggins *et al*, 1997). These mutations could be homozygous (carried on the two alleles) (Kim *et al.*, 1996) or missense gene mutations in cancers of the pancreas (Hahn *et al.*, 1996), biliary tract (Hahn *et al.*, 1998), colon (Eppert *et al.*,1996; Riggins *et al.*, 1996; Takagi, 1996) and in hepatocellular carcinoma (Yakicier *et al.*, 1999).

1.6 The p38 MAPK Pathway

While the Smad pathway is initiated by BMP-2/4 binding to the preformed receptor complexes BRIa and BRII, the p38 MAPK pathway is reported to be initiated in the presence of BMP-2 by receptor complexes formed after ligand binding: the non-canonical BMP-2 pathway. The ligand binds to the high affinity receptor type I, then the receptor type II is recruited to form the complex. The p38 pathway is one of the four subgroups of the MAPK pathways family that mediate cellular behavior in response to extracellular stimuli. Mammalian p38s respond to stimuli

such as heat, osmotic shock, inflammatory cytokines and growth factors (Zarubin and Han, 2005).

The role of p38 in cellular commitment has not been thoroughly investigated. Normally studies employ pharmacological approaches via p38 inhibitors with the aim of understanding the role of p38 MAPK in inflammatory process. Commitment studies have often yielded contradictory results. Weston *et al.*, (2003) studying p38 in primary limb mesenchymal and C2C12 cell cultures report that p38 inhibition promotes myogenesis in the later stages of lineage commitment. This contrasts with the results by Cabane *et al.*, (2003) who claimed that a functional p38 MAPK pathway is indeed required for terminal muscle cell differentiation of C2C12 cells; Zester *et al.*, (1999) working with myogenic L8 cells, raised the possibility of p38 MAPKs being important during skeletal muscle differentiation in the activation of muscle-specific genes. On the osteoblastic side, Gallea *et al.*, (2001) reported p38 activation by BMP-2, and that reduction of ALP expression occurred following inhibition of p38 MAPK in C2C12 precursors. Vinals *et al.*, (2002), reported contrasting results where inhibition of p38 produced a 9 fold increase in ALP activity of C2C12 cells compared with a 5 fold increase in the control. Lee *et al.*, (2002), reported that Runx2, a transcription factor target of TGFβ-1 and BMP-2 plays an essential role in C2C12 commitment and transdifferentiation to the osteoblastic lineage by mediating the blockage of myogenic differentiation and inducing osteogenic commitment. They also added that after TGF-β and BMP induction, the Smad and p38 MAPK pathways converge at the Runx2 gene to control mesenchymal precursor cell commitment.

1.7 The TGF-β Pathway

The TGF-β pathway that transmits signals through Smad 2 and 3 may play a role in transdifferentiation. This pathway is part of the TGF-β pathways superfamily and shares many

similarities with the BMP pathway. After ligand binding, the receptors phosphorylate Smads 2 and 3 that then bind Smad-4 and are translocated to the nucleus where they interact with several DNA-binding proteins. After, the complex is formed and binds DNA, it affects the expression of proteins including Plasminogen activator inhibitor type 1 (PAI-1), JunB, p21, type I collagen, Smad-7, p15, and c-Myc (Miyazawa *et al.*, 2002). It has been demonstrated that BMP-2 can also activate the TGF-β pathway by increasing the autocrine production of TGF-β 1 and 3 and of the related ligand Activin. Depending on the phenotype and the differentiation status of the cells being tested, BMP-2 treatment may enhance TGFβ-induced ALP activity (Zamurovic *et al.*, 2004)

1.8 Goals

1. To investigate the conditions of commitment and differentiation of adult mesenchymal precursors and envision their potential use in xenografts for tissue replacement.
2. To test the commitment of myoblastic C2C12 cells towards osteoblast formation during transdifferentiation and the potential reversibility of the system in culture under near to physiological conditions (no transfection and presence of serum).

1.9 Hypotheses

Under our culture conditions:

- C2C12 progenitors retain the ability to transdifferentiate to osteoblasts upon exposure to appropriate concentration of BMP-2.
- The Smads pathway including Smad-8, is elicited by BMP-2 exposure.

- The kinetics of the Smads and markers of transdifferentiation are consistent with the dogma (standard) reported in the literature.
- Other pathways beside the Smads pathway may contribute to the transdifferentiation of C2C12 progenitors to the osteoblastic lineage.

Chapter 2
Materials and Methods

2.1 Chemicals and Reagents

All chemicals and reagents were obtained from commercial sources and were cell culture tested. The sources will be indicated in each section. All reported concentrations are final unless specified. The sources of antibodies and their dilutions are reported on the table 2.1 below: Dilutions were empirically optimized.

2.2 Cell Lines and Culture Conditions

2.2.1 Cell Culture Conditions

The cell line C2C12 was chosen as cellular model (American Type Culture Collection ATCC CRL-1772 obtained from Mr. Andrew Ochalski, University of Ottawa). The cell is a murine mesenchymal progenitor capable of committing to the osteoblastic, myoblastic, chondroblastic and adipocytic (Fig 1.1) lineages and was used for studying transdifferentiation to the osteoblast lineage.

C2C12 cells were cultured at 37°C in an atmosphere containing 5% CO_2, in high glucose- DMEM, (Gibco) pH 7.4 with the following additions: 10% (v/v) heat-treated (to inactivate complement) FBS (HyClone); nucleosides (Sigma) adenosine (29.9uM), guanosine (29.3uM), cytidine (30.0 uM), uridine (29.8uM) and thymidine (9.9 uM); non essential amino acids (Hyclone, 0.1mM); L-glutamine (Hyclone, 2mM); sodium pyruvate (Hyclone, 1mM); sodium bicarbonate (Wisent, 17.8mM); the antibiotics gentamicin (Wisent, 1.078mM) and penicillin and streptomycin (Hyclone, 100 ug/ml each). Cells were regularly checked for potential mycoplasma contamination (MycoAlert®, Cambrex).

Antibody. Catalog number	Supplier	Application/Dilution	
		Immunofluorescence	Immunoblotting
pSmad-1,5,8 rabbit. 9511	Cell Signaling	1:50	1:1000
Smad-1,5,8 (N-18) goat. IgG sc-6031-R	Santa Cruz biotechnology	1:200	1:500
pSmad-1 goat. IgG sc-12353	Santa Cruz biotechnology	1:200	1:500
Smad-4 (H-552) rabbit. IgG sc-7154	Santa Cruz biotechnology	1:200	1:500
Smad-8 (R-64) rabbit. IgG sc-11393	Santa Cruz biotechnology	1:200	1:500
Smad-6 (H-150) rabbit. IgG sc-13048	Santa Cruz biotechnology	1:200	1:500
Smad-7 (H-79) rabbit. IgG sc-11392	Santa Cruz biotechnology	1:200	1:500
Myogenin (M-225) rabbit. IgG sc-576	Santa Cruz biotechnology	1:200	1:1000
Actin (I-19) goat. IgG sc-1616	Santa Cruz biotechnology	NA	1:1000
Wheat Germ Agglutinin (W849)	Invitrogen	1:500	NA
AlexaFluor 488nm donkey anti-goat IgG. A11055	Molecular Probes	1:500	1:500
AlexaFluor 488nm goat anti-rabbit IgG. A11008	Molecular Probes	1:500	1:500
Alexa Fluor® 488 donkey anti-goat IgG (H+L) A-11001	Molecular Probes	1:500	1:500
Goat anti-rabbit alkaline phosphatase IgG (H+L). 111-055-003	Jackson ImmunoResearch Laboratories, Inc.	NA	1:3000 1:5000
Donkey anti-goat alkaline phosphatase IgG (H+L). 705-055-003	Jackson ImmunoResearch Laboratories, Inc.	NA	1:3000 1:5000

Table 2.1. Antibodies. Antibodies used in this project, their dilutions and manufacturers.

2.2.2 Subculturing/Passaging

The cell stocks were grown in flasks (Falcon) or tissue culture dishes (BD Biosciences) and subcultured every second or third day as needed to avoid reaching more than 80% confluency. For subculturing the medium was removed from the flask or dish and the cell monolayer washed once with Ca^{2+} and Mg^{2+}-free PBS (PBS is sodium chloride 0.154M, sodium phosphate dibasic 5.6mM, potassium phosphate monobasic 1.02uM).

Trypsinization (disassociation and detachment of adherent cells, using the enzyme trypsin) was done by adding to the flask or dish enough 0.15% Trypsin-EDTA (Gibco) to cover the cells with a thin film, the flask was then incubated at 37°C for 2 minutes to allow cell dissociation. The cells were collected in 10%-FBS medium and centrifuged at 1000 rpm at 4°C for 5 minutes (Baxter Megafuge, Henaeus Instruments). The supernatant was discarded and the cell pellet was resuspended in fresh medium. The cells were counted on a hemocytometer (Hausser Scientific) using trypan blue (Gibco) exclusion procedure adapted from Invitrogen Corporation. Cells were typically seeded at a density of approximately 1.7 x 10^4 cells/ cm^2.

2.2.3 Treatments to Induce Commitment of C2C12

To induce commitment of the C2C12 cells towards the myoblastic lineage, the culture medium was replaced with a medium prepared as above except for a reduction in FBS from 10% to 5% (v/v). To induce commitment towards the osteoblastic lineage, the culture medium was replaced with the medium as above with 5% (v/v) FBS and the addition of recombinant human BMP-2 (rhBMP-2, Prepotech) to a concentration of 23nM (300ng/ml). Cells were kept under commitment conditions (BMP-2 presence) for periods of time ranging from 10 minutes to 6 days. At determined time points the cells were fixed or lysed for

experimentation. According to the manufacturer, reconstituted rhBMP-2 is stable for 69 hours (Peprotech, personal communication) at 37°C. Therefore, at day 3 the culture medium was replaced with fresh medium containing rhBMP-2. The effectiveness of the treatment was monitored by evaluation of alkaline phosphatase (ALP) activity.

2.3 Cell Proliferation

2.3.1 Population Size Estimation

Cell population sizes and growth were estimated using a protocol adapted from Rao and Otto (1992) in order to generate cell growth curves. Cells were seeded in flat bottom 96-well plates (TPP) at a density of approximately 1.7 x 10^4 cells/cm^2, incubated at 37°C and treated with 23nM rhBMP-2 after settling. At increasing times following exposure to the commitment conditions, cells were rinsed twice (200ul each time) without shaking with PBS containing Ca^{2+} and Mg^{2+} (0.9M $CaCl_2.2H_2O$ and 0.49M $MgCl_2.6H_2O$) Then 100ul of lysis buffer (69mM SDS in SSC (SSC is 0.15M NaCl, 15mM trisodium citrate, pH 7.0)) was added to the wells and the plates were stored at -70°C overnight to favor cell lysis. The following day, the plates were incubated at 37°C for 45 minutes and 100ul per well of a 7.5uM solution of Hoechst 33325 (Sigma) in SSC was added. Fluorescence was measured in a FLUOstar Galaxy Reader (BMG Labtechnologies) with an excitation wavelength of 320nm and an emission wavelength of 485nm. The relative fluorescence units are directly correlated to the number of cells.

2.3.2 Proliferation Rate and Index

To monitor cell proliferation rate, cells were stained with the lipophilic fluorescent membrane linker DiI, (Molecular Probes) prior to exposure to differentiating medium. DiI

binds to the cell membrane and is equally divided between the daughter cells upon cell division. At chosen time points, cells were trypsinized according to the subculturing protocol described in section 2.2.2. Cell suspensions were then pelleted by centrifugation at 1000 rpm for 5 minutes at 4°C, and washed with FBS-free medium (DMEM or α-MEM depending on the cell line). 1 x 10^6 cells were resuspended in 2ml of 0%-FBS medium with 2uM DiI and incubated for 20 minutes at room temperature with gentle agitation every 5 minutes. Cells were then pelleted by centrifugation and washed twice with 0%-FBS medium to remove the excess DiI, then resuspended in growth medium containing FBS. Cell concentration was determined for subsequent seeding and differentiation or commitment depending on the cell line. At various time intervals, cells were collected following trypsinization, washed with PBS and resuspended in an appropriate amount of 15mM BSA in PBS for subsequent analysis by flow cytometry (Beckman–Coulter Elite, Cytomics FC500 equipped with an Argon laser (488). Channels used for DiI were FL3 (605-635, Max excitation 536nm and Max emission 617nm). No fewer than 50,000 cells were analyzed (standard error 0.02%). In each division, cells lose half of the linker and therefore half of their fluorescence. Based on this observation the program ModFit (Verity Software House) can calculate the average number of generations, the proliferation rate and the proliferation index (Appendix C).

2.4 Protein Extraction and Quantification

2.4.1 Total Protein Extraction (RIPA)

Cells grown in 10 or 15 cm diameter tissue culture dishes were washed with PBS and lysed with 350-500ul (depending on dish size and protein concentration wanted) of radioimmuno precipitation assay (RIPA) buffer, pH 8.0, (17.2mM Nonidet-P40, 12mM

sodium deoxycholate, 3.4mM SDS in PBS without Ca^{2+} and Mg^{2+}) containing proteaseinhibitors (Sigma: 5.6mM PMSF, 1 mM sodium orthovanadate, 15.2nM aprotonin, and 11.3uM leupeptin). The dishes were then stored at -70°C overnight to favor cell lysis. The cells were then scraped in the lysis buffer, transferred into microfuge tubes and passed through a 26 gauge needle 10 times to shear the DNA. Following incubation on ice for 30 minutes the samples were centrifuged for 30 minutes at 14,000 rpm at 4°C. The supernatant was collected and an aliquot was used for protein quantification as per section 2.4.3.

2.4.2 Isolation of Nuclear Protein Fraction

Cells grown in 10 or 15 cm tissue culture dishes were quickly rinsed with Ca^{2+} and Mg^{2+} free PBS, after, 330 or 500ul of hypotonic buffer (enough to cover the cells) (10mM HEPES; 0.5mM KCl and 2mM MgCl in water) were added and distributed uniformly in the plate to lyse the cells without disturbing the nuclei. The dishes were kept on ice for 10 minutes, cells scraped into microfuge tubes and then centrifuged at 2500 rpm for 5 minutes at 4°C to separate the cytoplasmic (supernatant) and nuclear (pellet) fractions. The pellet was suspended in nuclear lysis buffer (20mM Tris-HCl,; 50mM NaCl, 8.5mM Nonidet P40, 12mM sodium deoxycholate, 17mM SDS and 1mM EDTA in water), passed trough a 26 gauge needle 10 times to shear DNA and then centrifuged at 14000 rpm for 30 minutes at 4°C. The supernatant corresponding to the nuclear fraction was stored at -20°C for protein quantification and experimentation. Protein concentration of the nuclear lysate was estimated as described below.

2.4.3 Protein Quantification

The concentration of the proteins from the supernatants collected in 2.4.1 and 2.4.2,

was determined by a dye binding principle assay (Bradford, M., 1976). 5ul of supernatant were added to 1995 uL of water, and 200 uL of Bio-Rad protein assay reagent. The absorbance of the solutions was measured at 596 nm on a diode array spectrophotometer (Hewlett Packard) against a blank solution. A standard curve for protein concentration was made using bovine serum albumin (BSA, Amaresco), and used as the standard for quantification.

2.5 Protein Electrophoresis and Staining

2.5.1 Gel Electrophoresis

Normalized protein lysate (~30ug protein) was mixed with 1/3 volumes of 6X loading buffer (Novagen) to a final concentration of 2X and was boiled for 3 minutes. The samples were loaded onto a 3.75% polyacrylamide-SDS stacking gel containing 0.1% SDS and electrophoresed through a 10% polyacrylamide-SDS denaturing separating gel with 0.1% SDS (Laemmli gel).

2.5.2 Coomassie Blue Protein Detection

Following electrophoresis, selected gels were stained with Coomassie blue to allow the visualization of bands indicating the protein separated on the gel. The protocol was adapted from Hames and Rickwood (1981). After separation of peptides by electrophoresis on SDS polyacrylamide gels, the gel was incubated overnight in staining solution (60uM Coomassie Brilliant blue R-250, 10% acetic acid, 25% methanol) followed by destaining with three changes of distaining solution (40% methanol, 7% acetic acid) until the background was low. After rinsing with water, the gel was mounted between pre-wetted

cellophane sheets in a frame (Bio-Rad) and allowed to dry under vacuum-free conditions at room temperature.

2.5.3 Ponceau Red Staining

Following transfer of the proteins from the gel to the nitrocellulose membrane (section 2.5.4.), the membrane was stained with Ponceau Red solution [0.26mM Ponceau S in 3% trichloroacetic acid and 3% sulfosalicylic acid. (Rokaw *et al.*, 1996)] for 5 minutes to verify protein loading and transfer. Once the protein bands were visualized, the membrane was rinsed in TBS (Sigma) (50mM Tris base, 150mM NaCl) until decoloration of the protein bands prior to antibody detection as per section 2.5.4.

2.5.4 Protein Transfer and Western Blotting (WB)

Following electrophoretic separation the proteins were transferred to a nitrocellulose membrane using the Mini PROTEAN3 system and POWERPAC 200 (all from Bio-Rad) set to a fixed voltage of 100v for 1h. The apparatus was disassembled and the membrane was washed in TBS containing 1% Tween 20 (Sigma, TBST) once for 5 min with gentle agitation.

For immunodetection the membrane was incubated with the selected primary antibody (diluted 1:500 to 1:1000 in 15mM BSA in TBST) overnight at 4°C with gentle agitation. After washing in TBST 3 times each 10min, the membrane was placed in a solution of the appropriate secondary IgG antibody conjugated to alkaline phosphatase (Jackson) (diluted 1:3000 to 1:5000 in 15mM BSA in TBST) for 1 hour at room temperature with gentle agitation and dark conditions. The membranes were then washed 3 times for

10min each in TBST. Detection of protein bands was made following addition of the alkaline phosphatase substrate, 9.2mM BCIP, (Amaresco) and 2.4mM NBT, (Amaresco) in ALP buffer [100mM Tris-HCl, 100mM NaCl, 5mM $MgCl_2$ (adjusted to pH 9.5 with NaOH)] at 37°C. The reaction was stopped by rinsing the membranes in a 0.83M acetic acid solution. As an alternative to the ALP detection procedure, a chemiluminescent method was also used. After incubating with the primary antibody and washing 3 times for 10 minutes with TBST, the membrane was incubated with the appropriate secondary IgG antibody (dilution 1:5000 in 15mM BSA in TBST) conjugated to HRP, (Santa Cruz) for one hour. The membrane was then washed in TBST 3 times for 10 min and once with TBS, and incubated for one minute in just enough Luminol reagent (Santa Cruz) to cover the membrane. The excess Luminol reagent was gently removed by shaking; the membrane was mounted in a Kodak cassette and exposed to Kodak Scientific Imaging Film X-OMAT® for an appropriate length of time. The exposed film was processed through developing and fixing solutions (Kodak).

For both procedures, the constitutively expressed housekeeping protein actin was used as loading control (Sunesen *et al.*, 2003). The detection of the proteins for each WB required a different exposure time to substrates. Some blots were scanned and enhanced for better view. Therefore blot's bands can be compared within blots but not with bands of other blots.

2.5.5 Densitometric Analysis

For relative quantification, some WB membranes were scanned using a Scanjet 4400c (Hewlett Packard) scanner. Densitometric analysis of the protein bands was performed by using Gel-Pro Analyzer 3.1 software (Media Cybernetics).

2.6 Microscopy

2.6.1 Immunomicroscopy

Cells grown on coverslips were rinsed quickly without shaking two times with PBS containing Ca^{2+} and Mg^{2+} and fixed by incubation with 1.33M paraformaldehyde (prepared in the same buffer) for 30 minutes at room temperature. Cells were rinsed twice without shaking in PBS with Ca^{2+} and Mg^{2+} , permeabilized with 3.4mM Triton X-100 (Sigma) in PBS containing Ca^{2+} and Mg^{2+} for 10 minutes at room temperature and then washed two times with PBS containing Ca^{2+} and Mg^{2+}. Non-specific sites were blocked by incubation with 15mM BSA in PBS with Ca^{2+} and Mg^{2+} for 1 hour prior to addition of the corresponding primary antibody (no rinsing is needed at this step) diluted in 15mM BSA in PBS with Ca^{2+} and Mg^{2+} and incubation overnight at 4°C with gentle agitation. Following quick rinsing two times with PBS with Ca^{2+} and Mg^{2+} the sample was incubated with a 1:5000 dilution of the corresponding secondary antibody (Alexa 488-conjugated, Molecular Probes) solution prepared in 15mM BSA in PBS with Ca^{2+} and Mg^{2+} for 1 hour at room temperature without agitation. Coverslips were rinsed quickly three times with PBS with Ca^{2+} and Mg^{2+}, and mounted onto glass slides for microscopy and observation using an appropriate filter (B2A, Excitation 450-490 nm, Emission 520 nm) and epifluorescence equipped Olympus microscope with Image Pro Plus imaging software (MediaCybernetics). Controls for nonspecific secondary antibody binding were performed.

2.6.2 Nuclear Staining With DAPI

Cells cultured on coverslips (Bellco Glass) were fixed and permeabilized as in the immunofluorescence protocol and exposed for 10min at room temperature to 0.1uM DAPI in

PBS with Ca^{2+} and Mg^{2+} (Vector Labs). DAPI is known to bind DNA. The cell preparations were then quickly rinsed twice with PBS with Ca^{2+} and Mg^{2+}, examined and photographed under UV illumination with a Olympus microscope equipped with epifluoresence and the corresponding fluorochrome filters (UV-1A, Excitation 365 nm, Emission 400 nm) to locate the nuclei and as a control to check for possible Mycoplasma contamination.

2.6.3 Confocal Microscopy

Confocal microscopy was performed to precisely determine the sub- localization of some proteins. Cells were cultured, rinsed, fixed, permeabilized, non-specific sites were blocked, then cells were incubated in the corresponding primary and fluorescein-conjugated secondary antibodies as described in immunomicroscopy, section 2.6.1. The coverslips were then mounted with Vectashield (Vector Labs) hardening mounting medium, cells facing down on a microscope slide and left at 4°C in the dark overnight to allow mounting medium hardening. Images were taken with a LSM-410 Zeiss (Carl Zeiss, Thornwood, NY) inverted laser scanning microscope. Approximately 12 optical sections (1μm thick) of the specimen were taken. The confocal apparatus was set to generate an image from which 50% of the light came from an optical slice 1μm in thickness. Optical sections of labeled slices were obtained using the 488 nm excitation wavelengths (pass band filter 485/20) and for the emitted fluorescent light a 515-540 band pass filter was used. The ImageJ 1.37v software (National Institutes of Health, USA) was used to set the microscope parameters and generate the images.

2.7 Alkaline Phosphatase Measurements

2.7.1 Cellular Alkaline Phosphatase Activity Estimation

A protocol to measure the relative variation of cellular ALP activity was designed based on the work of Jorgensen *et al.*, (2004) and the LabAssay ALP kit from Wako. Cells were cultured under the desired conditions, in our case in 24-well plates (2 cm^2) at a cell density of 1.7 x 10^4 cell/cm^2. After incubation and treatment the cells were rinsed twice with PBS without Ca^{2+} and Mg^{2+}. After followed the addition of 450ul of alkaline buffer (2mM $MgCl_2$, 0.1M carbonate buffer, in water. pH 9.8) and incubation at 37°C for 10 min, then cells were scraped from the dish and transferred to a microtube, and pipetted 10 times until complete suspension. Following centrifugation at 2000rpm, 7 minutes at 4°C the supernatant (cell lysate) was stored at -20°C or processed as follows. 70ul of cell lysate was transferred into a well of a 96 well plate and 70ul of substrate solution (6.7mM of pNP, Fluka) was added. The plate was incubated for 30 min at 37^0C and the reaction stopped by the addition of 70ul of 2N NaOH (Sigma). The absorbance at 405-410nm was read. To convert the ALP activity to nmol of pNP per mg of protein, the protein content of cell lysates was estimated as in section 2.4.3. An ALP standard curve was generated and the cell populations under the different treatments at time points of interest were calculated.

2.7.2 Cellular Alkaline Phosphatase Staining

Detection of ALP by staining was performed using a protocol adapted from The Miller Lab, available on line at http://www.fhcrc.org/science/labs/miller/hpapstain.html and Chemicon's Alkaline Phospatase Detection Kit Application. Cultured and treated cells were washed three times with PBS with Ca^{2+} and Mg^{2+}, fixed with 1.33M paraformaldehyde (Sigma) in PBS with Ca^{2+} and Mg^{2+} for a maximum of 2 minutes to avoid loss of ALP

activity. Then ALP buffer solution (100Mm Tris pH 8.5, 100Mm NaCl, and 50 Mm $MgCl_2$) containing 1x concentration of NBT (100X stock: 61mM NBT, in 70% dimethylformamide (Sigma) and 30% water) and 1X concentration BCIP, (100X stock: 23mM BCIP in water) was added. Cells were incubated at 4°C in the dark overnight with gentle shaking, and pictures taken the following morning. This method was used in order to test whether all cells in the population were expressing ALP.

2.7.3 Alkaline Phosphatase Back-extraction.

After ALP staining, the colored substrate was extracted with 100% DMSO. 250µl of DMSO per cm^2 of cultured area were added with gentle shaking for 24h at room temperature. After the DMSO was collected, 100ul were transferred to flat bottom 96-well plates and the absorbance read at 320nm. This methodology was developed in our laboratory and still needs to be optimized in the high activity range. Therefore the results should be considered semi-quantitative or trend indicative. Proper validation will be part of the future work. This procedure was experimented as a complement to the ALP staining in order to obtain semi quantitative figure that could compare with the PNP-based ALP activity estimation.

2.8 Inhibition of Signaling Pathways

2.8.1 Inhibition of the TGF-β Pathway

The TGF-β pathway was blocked with the specific inhibitor SB431542 (Sigma) at a 10µM final concentration in the medium for 30 minutes at 37°C (Inman *et al.*, 2002. Maeda *et al.*, 2003). Following the addition of 23nM BMP-2 (Maeda *et al.*, 2004), cells were cultured for six days as described in the Cell Culture Conditions section. The inhibitor and

the morphogen were renewed in fresh medium at day 3. Immunofluorescence estimation, ALP staining or protein extraction were performed as required.

2.8.2 Inhibition of the p38 Pathway

The p38 pathway was selectively blocked with 10μM SB202190 (Sigma) final concentration (Degousee *et al.*, 2003; Weston *et al.*, 2003). Cells were cultured for six days as described in the Cell Culture Conditions section. The inhibitor and the morphogen were renewed in freesh medium at day 3. ALP activity was evaluated using both procedures: directly by substrate color measurement and indirectly by color extraction.

2.9 Cell Starvation

Cells were cultured under subculturing conditions for 24h, after medium was removed and the cells rinsed with PBS twice. Then 0.5%-FBS medium was added to the cells that were maintained under those conditions for increasing periods of time. The cells were further processed for experimentation or the medium was replaced with FBS-supplemented medium until ready for experimentation processing.

Chapter 3
Results

3.1 Characterization of the Cell Model

The C2C12 cell line was chosen as a model for this study. It is a multipotential mesenchymal precursor-like, derived from adult mouse muscle, capable of committing to the myoblastic, osteoblastic, chondrogenic or adipogenic lineages, and is a well established model for the study of cellular transdifferentiation to the osteoblastic lineage. The cell model was initially characterized to establish proper culture conditions for osteoblast commitment and differentiation based upon four parameters: proliferation, morphology, osteoblast differentiation as monitored by the expression of alkaline phosphatase and the simultaneous repression of the myoblast phenotype as assessed by myogenin marker expression.

Since morphology alone is not sufficient to identify a cellular phenotype, the use of markers known to be characteristic of a phenotype is a common tool to confirm cellular identity. In this project, alkaline phosphatase an enzyme known to be well expressed in osteoblastic cells and myogenin known to be well expressed in myoblastic cells were used as markers for each lineage. In this project, "myoblastic" is related to C2C12 progenitors not exposed to BMP-2 while "osteoblastic" relates to the cells exposed to 300ng/ml of BMP-2.

3.1.1 Cell Proliferation

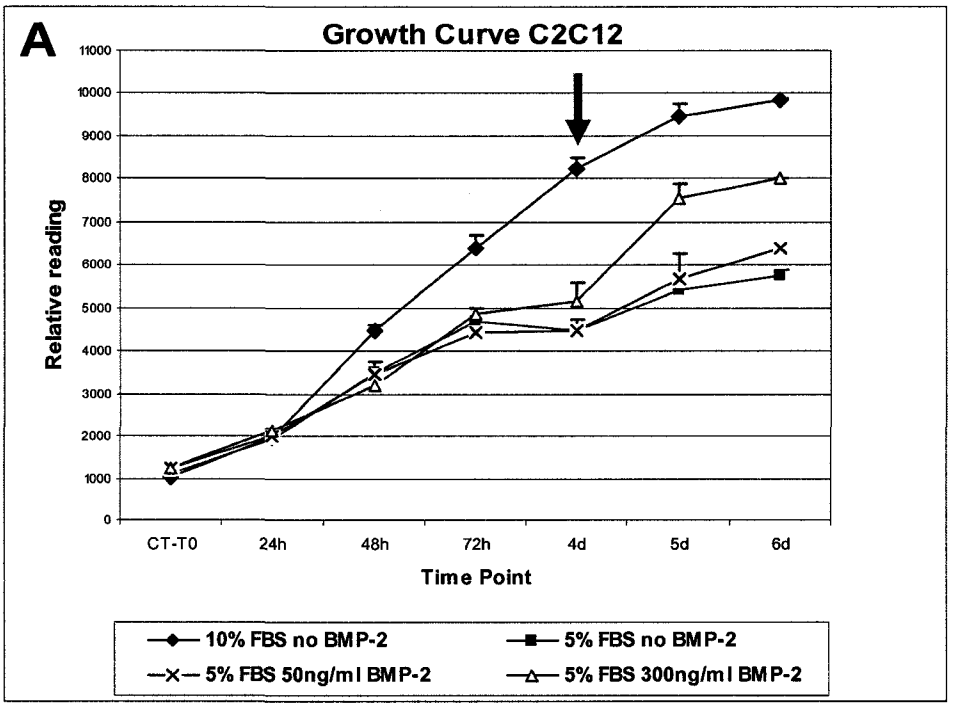
The growth of C2C12 cells was monitored under pluripotency conditions (10% FBS) and under commitment conditions (FBS reduced to 5%) with BMP-2 concentrations of 0, 50 and 300ng/mL. The growth of cells in 10% of FBS is higher than in all other treatments and continues up to 5 days before experiencing a slight decline in growth rate. However cells

grown in 5% FBS without or with BMP-2 had a reduced growth rate reaching a plateau by day 3. At this time, the addition of BMP-2 at a concentration of 300ng/mL in fresh medium containing 5% FBS was able to boost the growth rate (Fig 3.1A). BMP-2 in solution at 37°C is only active for 3 days (Preprotech, personal communication). Based upon this data and the need to maintain the concentration of BMP-2 for morphogenesis, the decision was made to routinely change the medium and renew the treatment at day 3 for all cultures where comparisons were made between treatments with and without morphogen in 5% FBS. As shown in Figure 3.1B, growth continues without a plateau.

3.1.2 Cell Differentiation: Morphology

The morphology of the cells provides important clues to cellular differentiation. After the C2C12 cell culture attains confluency, cells committing to the myoblastic fate (default status) acquire a very distinct spindle shape while cells committing to the osteoblastic lineage upon BMP-2 exposure take a polygonal shape (Katagiri *et al.*, 1994).

Confluent C2C12 cells cultured in 5% FBS without BMP-2 show typical oriented and elongated spindle-shaped cells, a morphology associated with commitment and progress of pre-myoblasts towards their default terminal phenotype (Fig 3.2, A and C). The cells fuse to form multinucleated syncytia (blue arrow). Cells seem also to commit to the myoblastic phenotype if left in 10%-FBS medium long enough to reach over-confluency (culture state passed 100% confluency where the cells reduce their size to accommodate as many cells as possible in a monolayer). Lack of contact inhibition in the myoblastic cells is illustrated by the formation of cell multilayer: 2 or more cell layers stacked upon each other (blue area). The spindle-shaped cells are found in a higher plane on the upper layer and may appear out of focus in the micrograph.



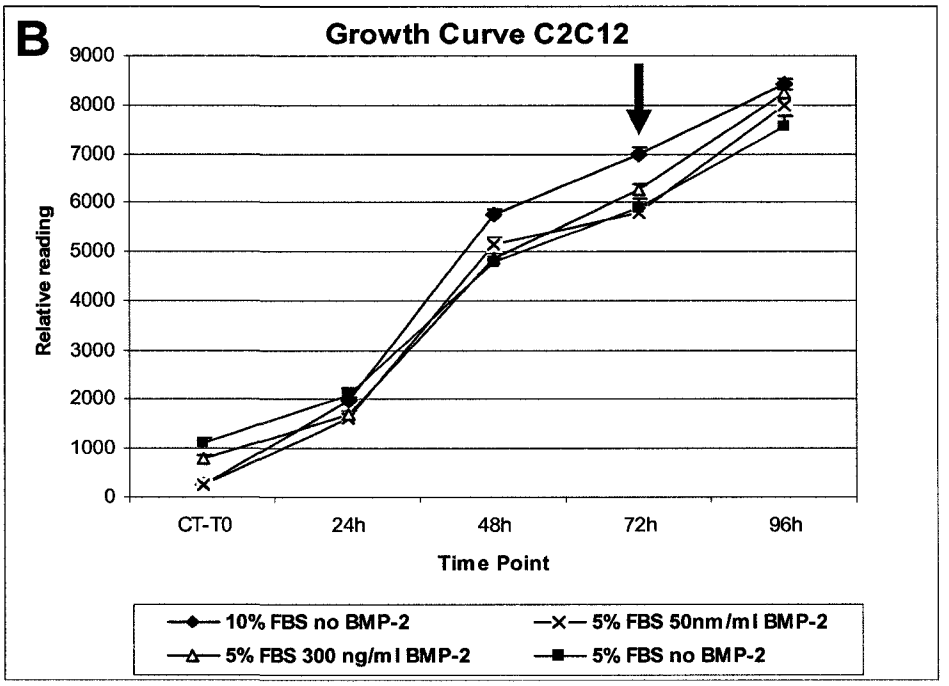


Figure 3.1 Cell proliferation under different culture conditions. C2C12 cells were cultured and proliferation monitored over the time periods indicated as described in Materials and Methods .The red arrow indicates the time at which the medium was replaced (see text). Each point represents the average of 5 replicates with the standard error shown. The error bars are shown in each time point and are sometimes difficult to see since the error was minimal.

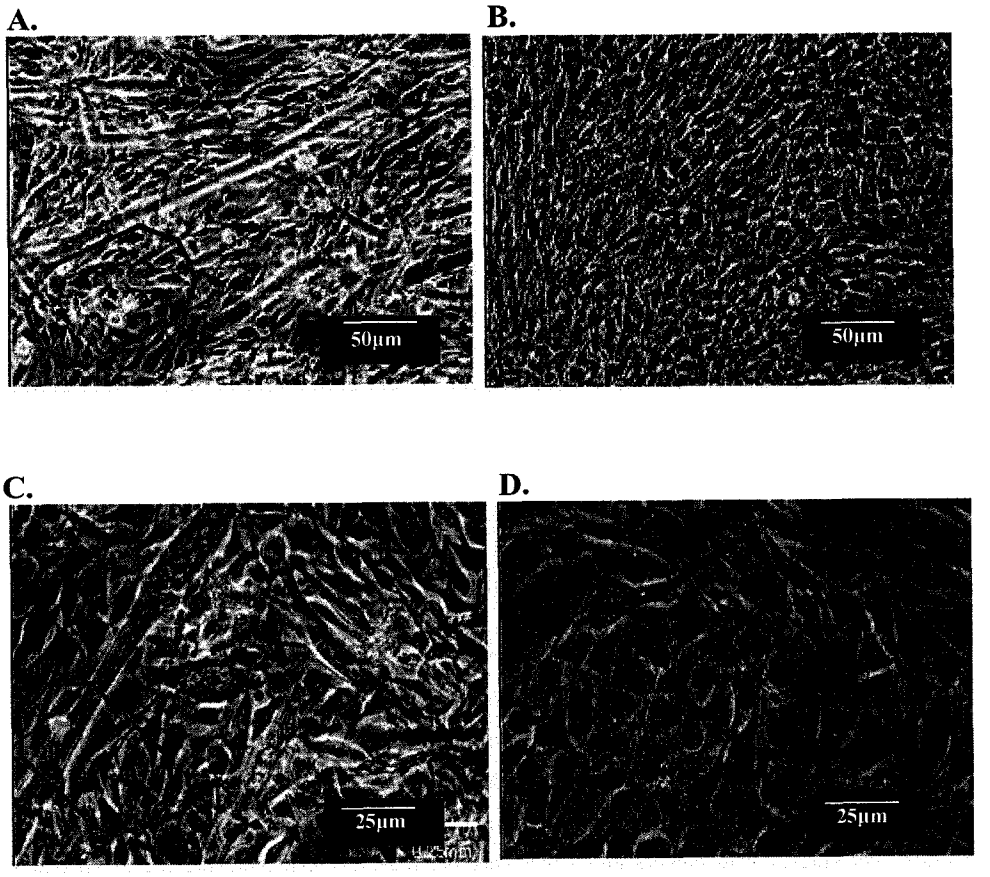


Figure 3.2. Morphology of C2C12 cells from confluent cultures. C2C12 mesenchymal progenitors were cultured until 6 days under myogenic commitment conditions (A and C) and osteoblastic commitment conditions (B and D). Then micrographs were taken as per section 2.6.1 of Materials and Methods. The arrows point at a multinucleated syncytia. The areas encircled in blue show sections of the micrographs where cell multilayer is present. Shown are representative examples of at least duplicates.

Cells cultured in 5%-FBS supplemented medium in the presence of 300ng/ml of BMP-2 for the same time period (Fig 3.2 B and D) show a completely different appearance. A polygonal morphology indicative of osteoblast phenotype is dominant at 6 days and cell multilayer is not as evident if present at all. The different morphologies observed especially in the myoblastic population may be due to heterogeneity that will be examined in more detail in later sections.

Under our culture conditions, C2C12 progenitors retain the ability to transdifferentiate to osteoblasts upon exposure to 300ng/mL BMP-2 as assessed by cellular morphology.

3.1.3 Osteoblastic & Myoblastic Status during Commitment and Differentiation

Changes to the morphology of C2C12 cells is insufficient to determine osteoblastic differentiation status upon exposure to BMP-2, thus alkaline phosphatase (ALP) activity was assessed. ALP is a widely accepted osteogenic marker. As a negative control for the osteoblastic lineage, myogenin a known muscle marker was monitored as well.

Alkaline phosphatase is a hydrolase that dephosphorylates numerous substrates. An increase in ALP activity is commonly accepted as a mark to identify bone formation (osteogenesis), and osteoblastic differentiation (Katagiri *et al.* 1994; Hsu and Tsai, 1997). Myogenin, a member of the MyoD family of proteins is a specific marker for skeletal muscle (Messina *et al.*, 2005). The default lineage for the C2C12 progenitors is myoblastic and myogenin expression was monitored by WB or immunofluorescence as a negative control for osteoblastic lineage progression.

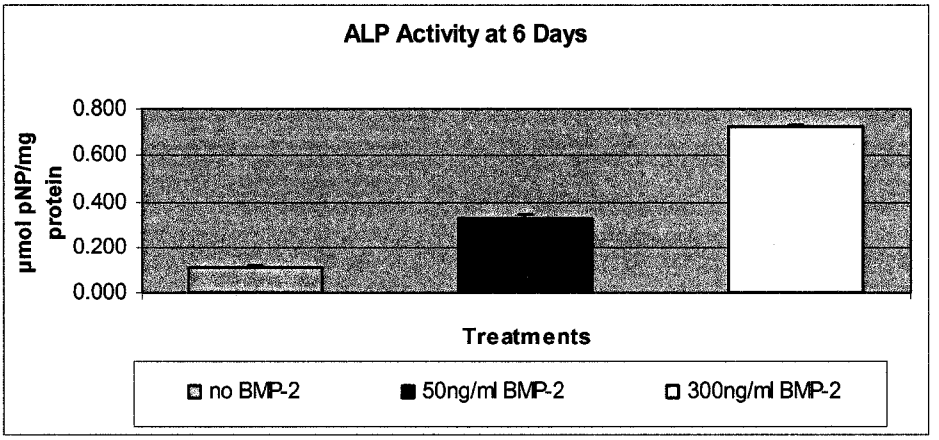
3.1.3.1 ALP Activity: Response to Increasing Doses of BMP-2

ALP activity increases with increasing doses of BMP-2. This was demonstrated by using several different methodologies (Fig. 3.3). Exposure of C2C12 to BMP-2 to induce osteoblast differentiation produces a significant increase in ALP specific activity (up to 0.72 ± 0.04 μmol p-NP/min/mg protein) when compared to the control (Fig 3.3A). A broad range of values have been reported in the literature for ALP activity following BMP-2 treatment. Our value is in the range of previously reported values of 0.4 and 9.0μmol p-NP/min/mg (Katagiri *et al.*, 1994 and Gallea *et al.*, 2001 respectively) for these cells.

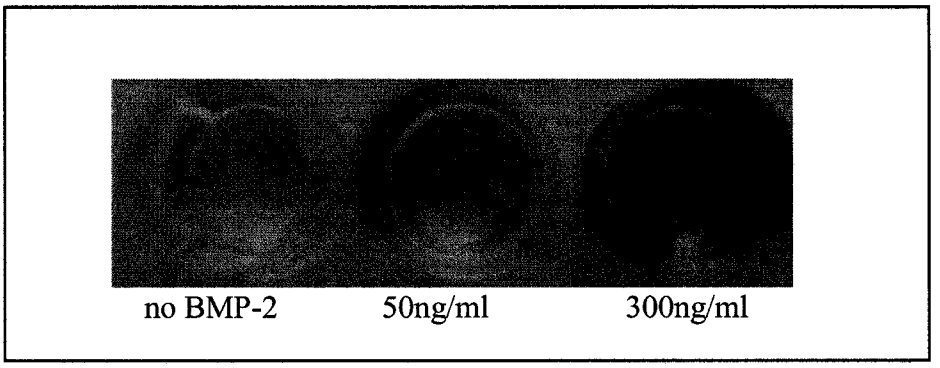
To confirm the change in ALP upon exposure to increasing doses of BMP-2, cells were directly stained using a different ALP substrate: NBT/BCIP (Fig. 3.3B). Low ALP expression is observed in the control cells (no BMP-2). At 50ng/ml BMP-2 ALP staining is higher than in the control while at 300ng/ml BMP-2 the highest staining can be seen. These results visually corroborate the measurements in figure 3.3A. Dark and light colored patches of cells are seen and especially noticeable at 50ng/ml, showing the uneven distribution of active ALP in the cells layer(s) and suggesting that commitment is uneven thus leading to heterogeneity. In order to obtain semi-quantitative data from this estimate of cellular activity of ALP with the NBT/BCIP substrate, back-extraction of the stained wells as per Materials and Methods section 2.7.3 was performed. As expected, (Fig 3.3C), when BMP-2 is present at 50 or 300ng/ml ALP activity increases.

Based upon these results, BMP-2 at a high dose (300ng/ml = 23nM) was used to initiate the osteoblastic phenotype. This dose is commonly used to induce C2C12 cells to form osteoblasts (Katagiri *et al.*, 1994; Gallea *et al.*, 2001).

A.


ALP Activity at 6 Days
µmol pNP/mg protein
0.800
0.600
0.400
0.200
0.000
Treatments
no BMP-2
50ng/ml BMP-2
300ng/ml BMP-2

B.


no BMP-2
50ng/ml
300ng/ml

C.

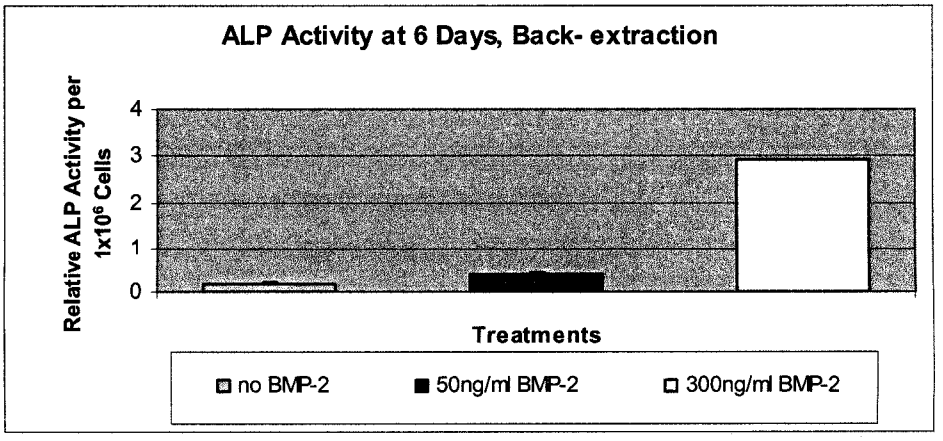

ALP Activity at 6 Days, Back- extraction
Relative ALP Activity per 1x10⁶ Cells
4
3
2
1
0
Treatments
no BMP-2
50ng/ml BMP-2
300ng/ml BMP-2

Figure 3.3 Changes in ALP activity during commitment.

A. C2C12 cells were cultured for 6 days in 5% FBS in 0, 50 or 300ng/ml BMP-2. ALP activity was estimated in cell extracts with pNP as the substrate as described in Material and Methods, Section 2.7.1. The standard error of the replicates is indicated. The control is the average of 2 replicates while the BMP-2 treatments are the average of 6 replicates.

B. C2C12 cells were cultured in 24-well plates for 6 days in 5% FBS with 0, 50 or 300ng/ml BMP-2. ALP activity was estimated following fixation and staining as described in Materials and Methods, Section 2.7.2. Exemplar is representative of duplicates.

C. C2C12 cells were cultured as in B. ALP activity was assessed by back-extraction of the ALP stain as per Materials and Methods Section 2.7.3. The standard error of the triplicates is indicated.

The uneven distribution of ALP staining observed in Fig 3.3B suggested that the cells are not uniformly responding to BMP-2. A closer examination of the ALP-positive cells was undertaken by standard light microscopy (Fig 3.4). As can be seen in figure 3.4, ALP is expressed in C2C12 cells. The dark areas correspond to high ALP activity areas. Some cell patches (yellow arrows) remain ALP activity free showing in detail the uneven distribution of the ALP as first illustrated in figure 3.3B. Based on ALP dose response to BMP-2 it can be stated that a high level of BMP-2 signal is necessary to elicit major ALP response. It is also clear that not all cells respond to the signal (Fig 3.3, 3.4).

In summary, exposure to the morphogen BMP-2 demonstrates that the morphological changes described in section 3.1.2 that accompany transdifferentiation from myoblasts to osteoblasts correlate with increases in ALP activity. In addition, we observed a differential response of the cell population following BMP-2 exposure revealing the heterogeneity of the cell population in terms of differentiation capabilities.

3.1.3.2 Myogenin expression: Dose Response to BMP-2 Exposure

Myogenin is a marker specific for skeletal muscle and its expression should decline during transdifferentiation. The level of myogenin was assessed by WB analysis and immunofluroescence.

At day 3 myogenin is expressed at similar but relatively low levels under both myogenic and osteogenic conditions (Fig 3.5) as determined by WB. In the myoblastic cells at day 6 the level of myogenin is higher than at day 3. In contrast at day 6 the expression of myogenin in the osteoblastic cells remains low. As only two replicates were performed, a precise estimate of the level of myogenin is not presented. This result is consistent with the known function of BMP-2 in repressing myogenin expression during osteoblast commitment

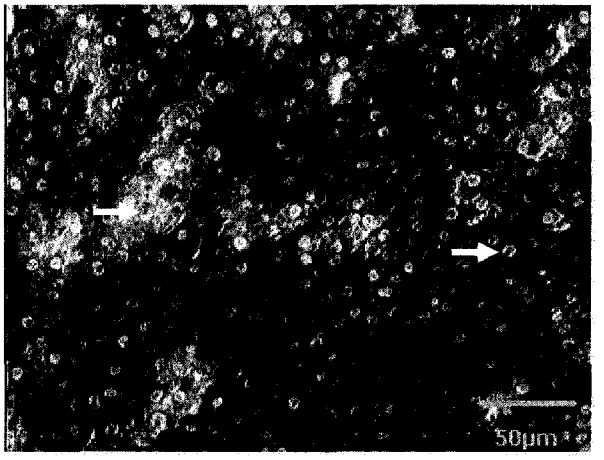


Figure 3.4. ALP expression by staining, cell field. ALP was stained in C2C12 treated with 300 ng/ml BMP-2 for 6 days as per Materials and Methods section 2.7.2. The dark coloured areas depict a good level of ALP activity, the white ones (nuclei and patches indicated by the arrows) represent little or no ALP activity expression. Micrographs were taken in duplicate at different BMP-2 exposure levels.

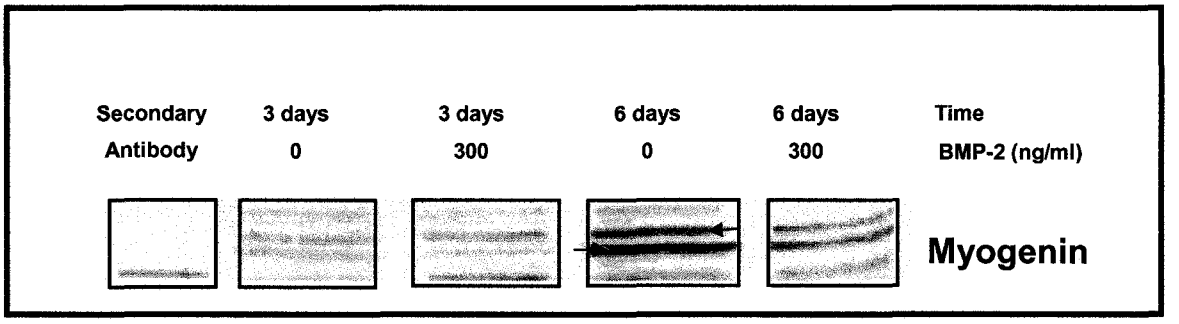


Figure 3.5. Changes in myogenin expression in response to commitment. C2C12 cells were cultured in 5% FBS supplemented with 0 or 300ng/ml of BMP-2 for 3 or 6 days. Cells lysates were prepared, and then 30ug of protein extract were electrophoresed through SDS/polyacryamide gels, transferred to membranes and probed with an anti-myogenin antibody as described in Materials and Methods section 2.5. For unspecific binding, cellular lysates from all treatments were processed with the secondary antibody alone; results were the same for all treatments. One of the secondary control lanes is included as a sample. Duplicate experiments were performed. The proteins were processed on the same membrane except for the secondary antibody control. Complete blots can be seen in appendix A. The arrows show the location of the phosphorylated (red arrow) and unphosphorylated (black arrow) myogenin.

(Katagiri *et al*, 1997; Lee *et al*, 1999). These results also show that myogenin is still present in the cell population advancing towards the osteoblastic lineage (3 and 6 days, 300ng/ml BMP-2).

The antibody used in these experiments, anti-myogenin (M-225), detects myogenin (black arrow) with an apparent molecular weight of 36kD and an additional slower migrating protein of higher molecular weight. This additional band (red arrow) may be phospho myogenin. Myogenin has been reported to be phosphorylated at threonine 87 (Blagden *et al.*, 2004) and other sites (Zhou and Olson, 1994). Katagiri *et al.*, 1997 working with C2C12 cells reported myogenin detection as two bands, the upper one being the hyperphosphorylated form and the lower one the unphosphorylated form. The antibody used (M-225), detects two bands in several cell lines including rat muscle (Santa Cruz, personal communication).

Immunofluorescence was used as an independent method to estimate myogenin levels at day 6 following the addition of BMP-2 (Fig 3.6). Cultures grown for 6 days in the absence of BMP-2 (Fig 3.6A) express high levels of myogenin as expected. The protein appears in the cells cytoplasm and/or the nucleus depending on the age of the culture. In contrast, cultures grown for 6 days in the presence of 300ng/ml of BMP-2 show lower and more diffuse fluorescence with the signal mainly located in the cytoplasm (Fig. 3.6B). Part of the signal is due to non-specific labelling with the secondary antibody (Fig. 3.6C).

The two results using the anti-myogenin antibody demonstrate that myogenin expression in cells that are not treated with BMP-2 increases overtime while in cultures treated with 300ng/mL BMP-2 the levels of myogenin do not increase as much but the protein remains expressed. Since an increase in ALP activity level at a treatment dose of 50ng/ml of BMP-2 was observed, the possibility that osteoblastic commitment would be

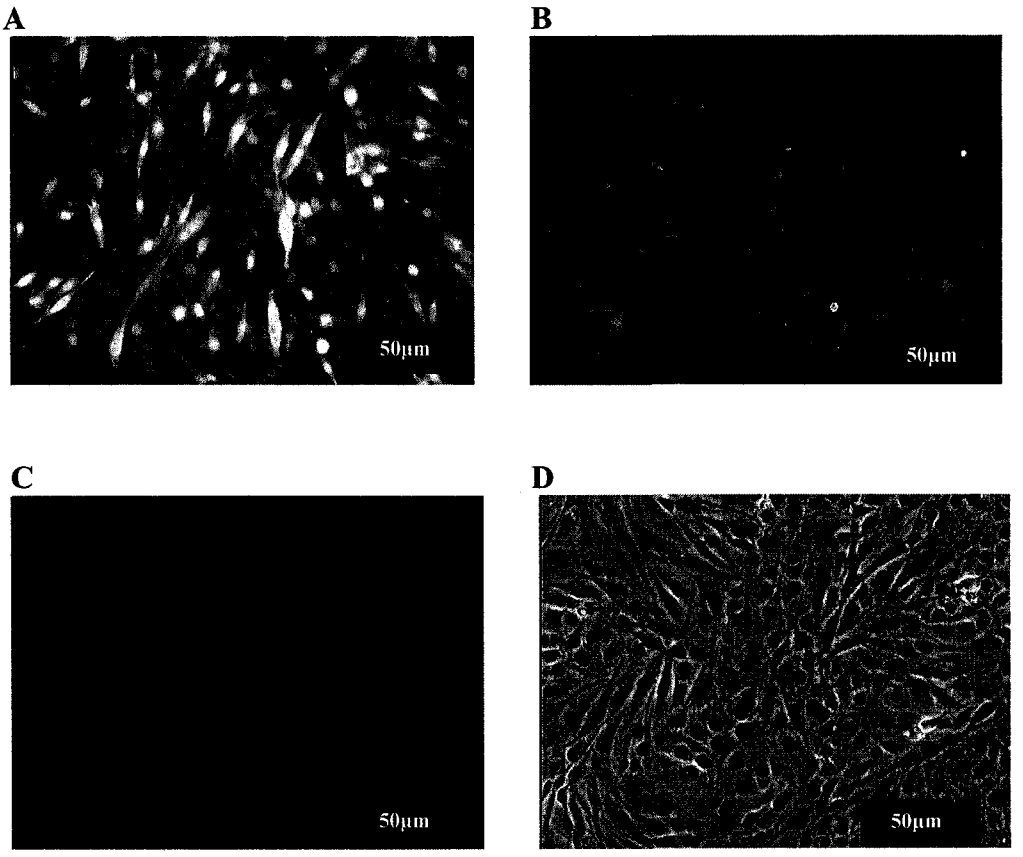


Figure 3.6. Myogenin expression during commitment as visualized by immunofluorescene.
Immunofluorescence of myogenin was determined using the anti-Myogenin M-225 antibody and an epifluorescence microscopy as described in Materials and Methods, section 2.6.1. Micrographs were taken under the same conditions to allow for comparison. (A) Myogenic cells, 6 days no BMP-2. (B) Osteogenic cells, 6 days 300ng/ml BMP-2. (C) Control secondary antibody. (D) Phase contrast of secondary antibody. Duplicate experiments were performed.

initiated at this dose was tested by immunofluorescence resulting in myogenin expression levels similar in controls and BMP-2 treated cultures at 6 days (not shown). In other words at a dose of 50ng/ml of BMP-2, osteoblastic commitment may have been induced but the signal was not sufficient to repress the myoblastic phenotype.

3.1.3.3 Intracellular Localization of Myogenin: Immunofluorescence Imaging

An attempt was made to confirm the presence of the marker and monitor its sublocalization during commitment and maturation of the osteoblastic lineage by using immunofluorescence.

Samples were processed at 3d (Fig 3.7A) and 6d (Fig 3.7B) following the addition of 300ng/mL of BMP-2. Unless specified, the intensity of the fluorescent signal has been adjusted for presentation purposes to facilitate the observation of the sublocalization of the marker. Therefore, the micrographs shall be only interpreted in terms of qualitative sublocalization of the myogenin but not as the quantitative expression of the protein.

At 3 days in culture, myogenin is expressed under myogenic and osteogenic conditions (Fig 3.7A). Cells cultured under myogenic conditions show an uneven label distribution within the cell with rare sublocalization into the nucleus. The cells cultured under osteogenic conditions show a pattern where the myogenin protein is expressed in the cytoplasm.

After 6 days under myogenic conditions the number of spindle-shaped cells has increased when compared to day 3 under osteogenic and myogenic conditions and 6 days under osteogenic conditions (Fig 3.7B.I). Also two characteristics of progress towards mature myotubules: syncytia (blue arrows) and multilayers are observed. Myogenin high expression is observed in a large number of cells in the cytoplasm and also in the nuclei of some cells.

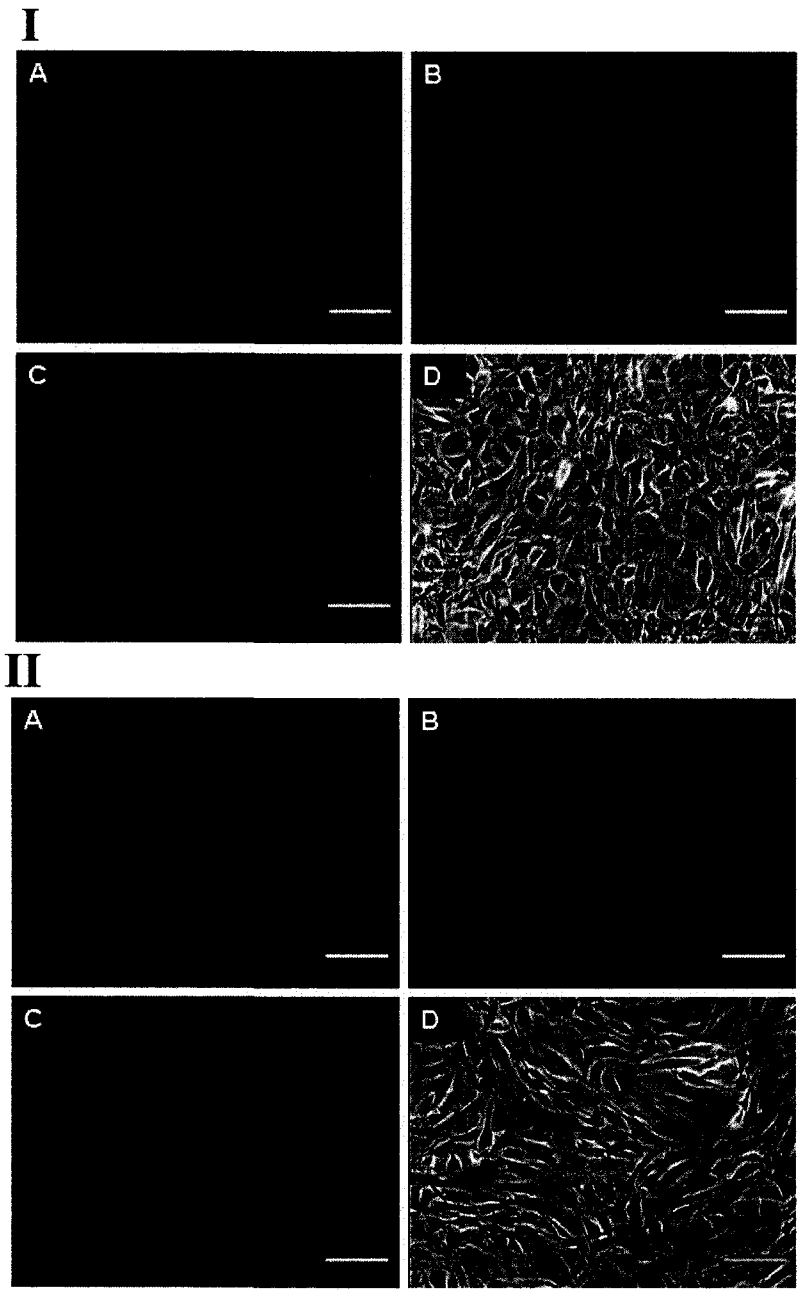


Figure 3.7A. Cellular sublocalization of myogenin at day 3. C2C12 were cultured under myogenic (**I**) and osteogenic (**II**) conditions for 3 days. Samples were prepared for microscopy as indicated in Materials and Methods section 2.6. The anti-myogenin antibody (M-225) was used. (A) DAPI nuclear staining; (B) immunolabeling of myogenin(s); (C) Superposition of (A) and (B) to demonstrate co-localization (yellow and/or orange color) at the nuclear level; (D) Phase contrast micrograph. The bars represent 50μm.

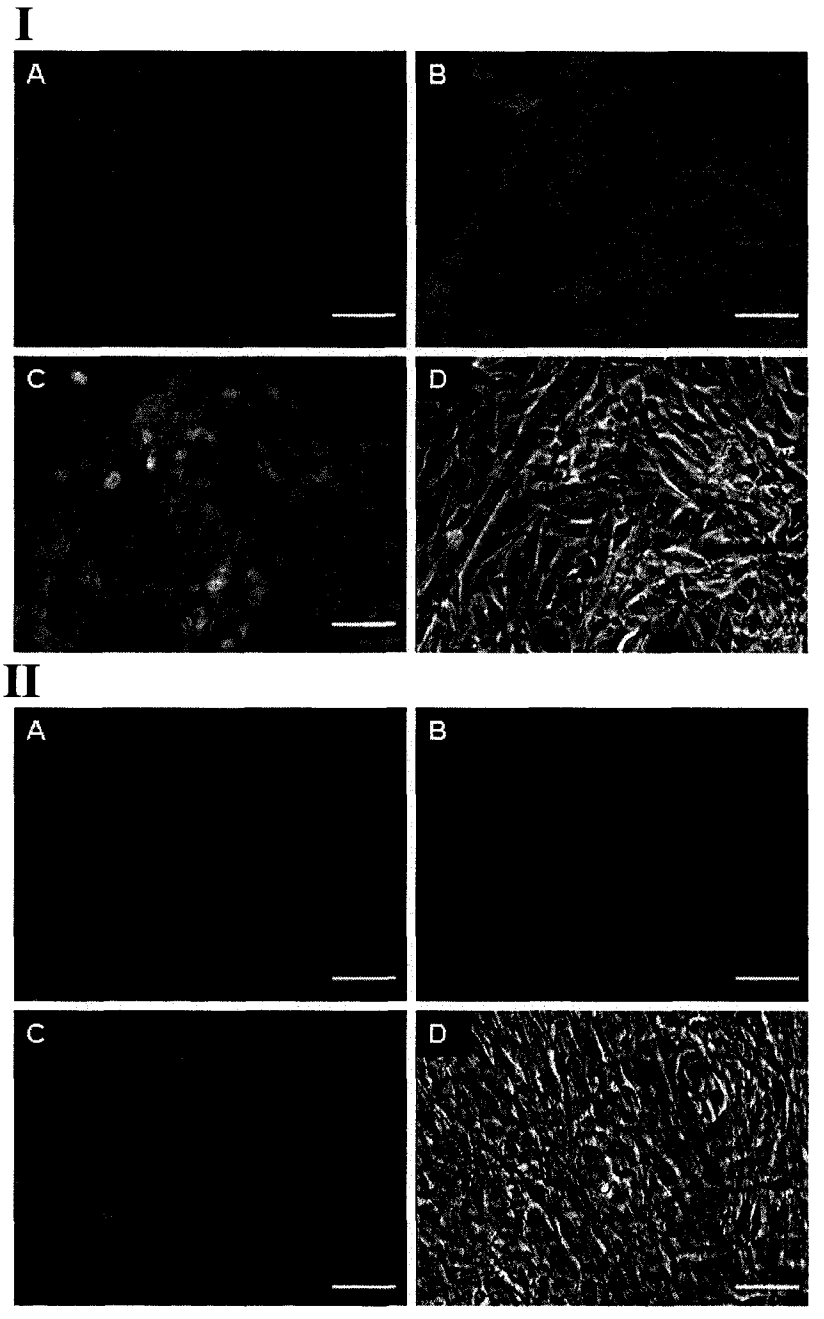


Figure 3.7B. Cellular sublocalization of myogenin at day 6. C2C12 were cultured under myogenic (**I**) and osteogenic (**II**) conditions for 6 days. Samples were prepared for microscopy as indicated in Materials and Methods section 2.6. The anti-myogenin antibody (M-225) was used. (A) DAPI nuclear staining; (B) immunolabeling of myogenin (C) Superposition of (A) and (B) to demonstrate co-localization (yellow and/or orange color) at the nuclear level; (D) Phase contrast micrograph. The arrows point at multinucleated syncytia. The lines represent 0.25μm.

Since myogenin is an activator of transcription, the subloclization in the nucleus is to be expected. Myogenin however, is still expressed at 6 days under osteogenic conditions (Fig 3.7B.I) it however, remains exclusively located in the cytoplasm; the morphology of the cells under osteogenic culture conditions is mainly cubical and multilayers were also observed. No syncitia were seen. These results confirm the WB analysis (Fig 3.5). Based upon these measurements of increased ALP activity and decreased myogenin levels (compared to the myoblastic fate) following commitment triggered by the addition of 300ng/ml BMP-2, we can conclude that this level of BMP-2 leads to the osteoblastic commitment in C2C12 cells. The modest but consistent expression of myogenin after 6 days of exposure to BMP-2 may result from the observed patchiness that, itself, may result from population heterogeneity. This point is addressed in the Discussion.

3.2 Commitment and Reversion upon Withdrawal of BMP-2

Commitment to the osteoblast fate follows the addition of 300ng/ml BMP-2. Next, the timing at which commitment to osteoblasts occurs and whether the continuing presence of BMP-2 was necessary to maintain the phenotypic transdifferentiation to osteoblasts was investigated. It is uncertain if C2C12 progenitors exposed to adequate amounts of BMP-2 and therefore induced to transdifferentiate lose the osteoblastic characteristics gained if the BMP-2 signal is taken away.

3.2.1 Monitoring ALP Activity

With the goal of investigating if and when reversion in ALP activity happens, several experiments were designed. In those experiments, BMP-2 was added to induce cellular

commitment and then withdrawn after increasing exposure periods of time, cells were then kept in culture for a total of 3 or 6 days (Total duration: BMP-2 exposure plus BMP-2 withdrawal). ALP and myogenin were monitored simultaneously to assess the cells fate.

There is a relation between the duration of exposure to BMP-2 and the activation of ALP - the longer the cells are exposed to the morphogen, the higher the ALP activity (Fig 3.8A). These data confirm that the C2C12 cells committed to the osteoblastic lineage upon BMP-2 exposure. Since the amount of BMP-2 that undergoes decomposition in the medium and the one that may be internalized by endocytosis and degraded in the cell are unknown, the quantity left at each time point under these conditions is also unknown. Therefore, we cannot assess if the progressive increase in ALP activity relates to the timing of the commitment among the cell population, the influence of the amount of BMP-2 remaining in culture (BMP-2 is being degraded) or a combination of these two factors.

When cultures were supplemented with BMP-2 for 6 or 24h and then cultured under BMP-2 free medium until day 3, ALP activity was higher than when measured immediately after the 6 or 24h of exposure (Fig 3.8B). This pattern suggests that the elicited pathway keeps functioning even after ligand removal. This may be due to a delay in ALP activity enhancement following commitment upon exposure to BMP-2. This experiment was then modified to include longer exposures to BMP-2 following the change of medium. Measurement of ALP activity at day 6 was implemented. By comparing the results from C (6 days) to those of A it appears that control cultures (CT-T0), despite of the complete lack of BMP-2, express some ALP activity. With longer exposure to BMP-2, to 6 or 24h before withdrawal (Fig 3.8C, Table 3.1 C), the level of ALP activity increases with time of exposure even though BMP-2 is withdrawn. Increasing the time of exposure does not further change

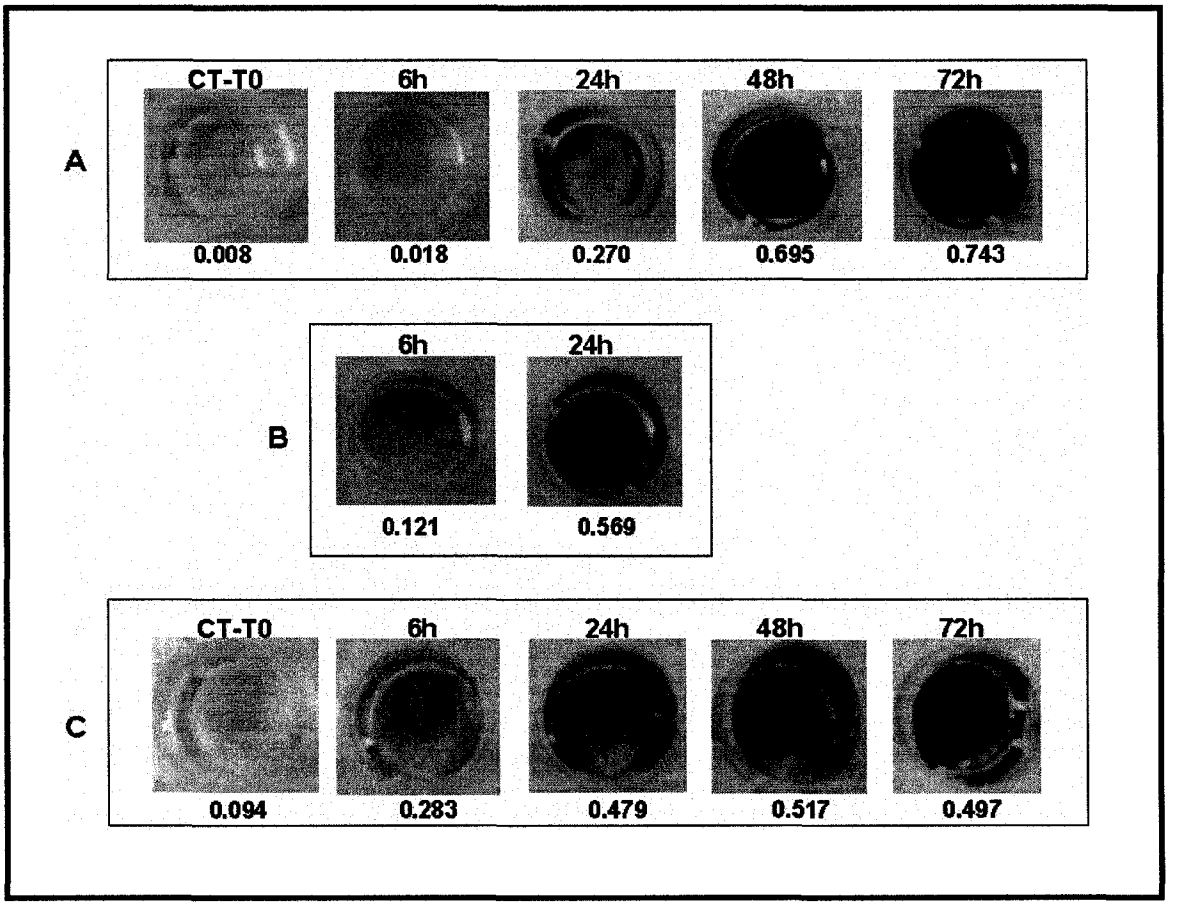


Fig. 3.8 Effect of BMP-2 withdrawal on C2C12 commitment as assessed by ALP activity.

Cells were cultured under osteoblastic commitment conditions and ALP activity was evaluated by staining with the BCIP substrate followed by back-extraction as described in Materials and Methods sections 2.7.2 and 2.7.3. (A) Cells were exposed to 300ng/ml of BMP-2 for increasing periods of time and tested immediately for ALP activity. (B) Cells were exposed to 300ng/ml of BMP-2 for 6 and 24 hours prior to exchange to fresh medium with 5% FBS, without BMP-2 and tested at day 3 for ALP activity. (C) Cells were exposed to 300ng/ml of BMP-2 for increasing periods of time, the medium was then changed to fresh medium with 5% FBS, without BMP-2 and all cells were tested for ALP activity at day 6. The figures underneath panels A, B and C are the average of triplicates (See Table 3.1). CT-T0 is control time zero.

Fig 3.8 Panel	BMP-2 exposure (h)	BMP-2 free time (h)	Time of Assay (h)	ALP Value average	Standard error
A					
	0	0	0	0.008	0.004
	6	0	6	0.018	0.004
	24	0	24	0.270	0.004
	48	0	48	0.695	0.006
	72	0	72	0.743	0.008
B					
	6	66	72	0.121	0.006
	24	48	72	0.569	0.006
C					
	0	0	144	0.094	0.007
	6	138	144	0.283	0.014
	24	120	144	0.479	0.009
	48	96	144	0.517	0.008
	72	72	144	0.497	0.012

Table 3.1. Effect of BMP-2 withdrawal on C2C12 commitment. The data derived from Fig. 3.8 are presented in a tabular form.

the level of ALP activity to any great extent and while the 48 and 72h data suggest that BMP-2 withdrawal may cause a drop in the ALP activity level when compared to samples stained right after BMP-2 exposure (Fig 3.8A), more replicate experiments are needed to confirm this observation. In panel A (Fig 3.8) the cells seem to be more committed at 24h than at 6h and ALP activity then seems to reach a plateau starting before or at 48h. The highest values for ALP activity measured in Fig 3.8A where activity is measured immediately following BMP-2 exposure (48 and 72h), are higher than the highest values for ALP activity measured in Fig 3.8C where activity is measured 3 to 4d following BMP-2 removal. This indicates that part of the population lost ALP activity upon BMP-2 removal and culture under BMP-2 free conditions or that undifferentiated cells have kept proliferating in the interval.

In summary, it can be concluded that cells commit to transdifferentiation to osteoblasts between 6h and 24h exposure to BMP-2. The continued presence of the morphogen is not required for the commitment of a large fraction of the cell population but may be necessary for a small portion of the culture. These results are novel.

In contrast to the pattern of ALP activity described above, myogenin synthesis should be repressed during transdifferentiation. Immunofluorescence was used to monitor myogenin expression and its sublocalization as depicted in figure 3.9.

3.2.2 Monitoring the Evolution of Myogenin Expression

Myogenin is well expressed and shows perinuclear sub-localization at 0, 6 and 24h exposure to BMP-2 (Fig. 3.9, IA, IB, and IC). At 48 and 72h (Fig 3.9 ID, IE) the protein remains mainly cytoplasmic but the perinuclear ring is not as heavily labelled as in IA, IB or IC. In the complete absence of BMP-2 for 6 days (Fig. 3.9 IIA), myogenin is strongly expressed in spindle-shaped cells and syncytia. A 6h exposure to BMP-2 does not change this pattern of

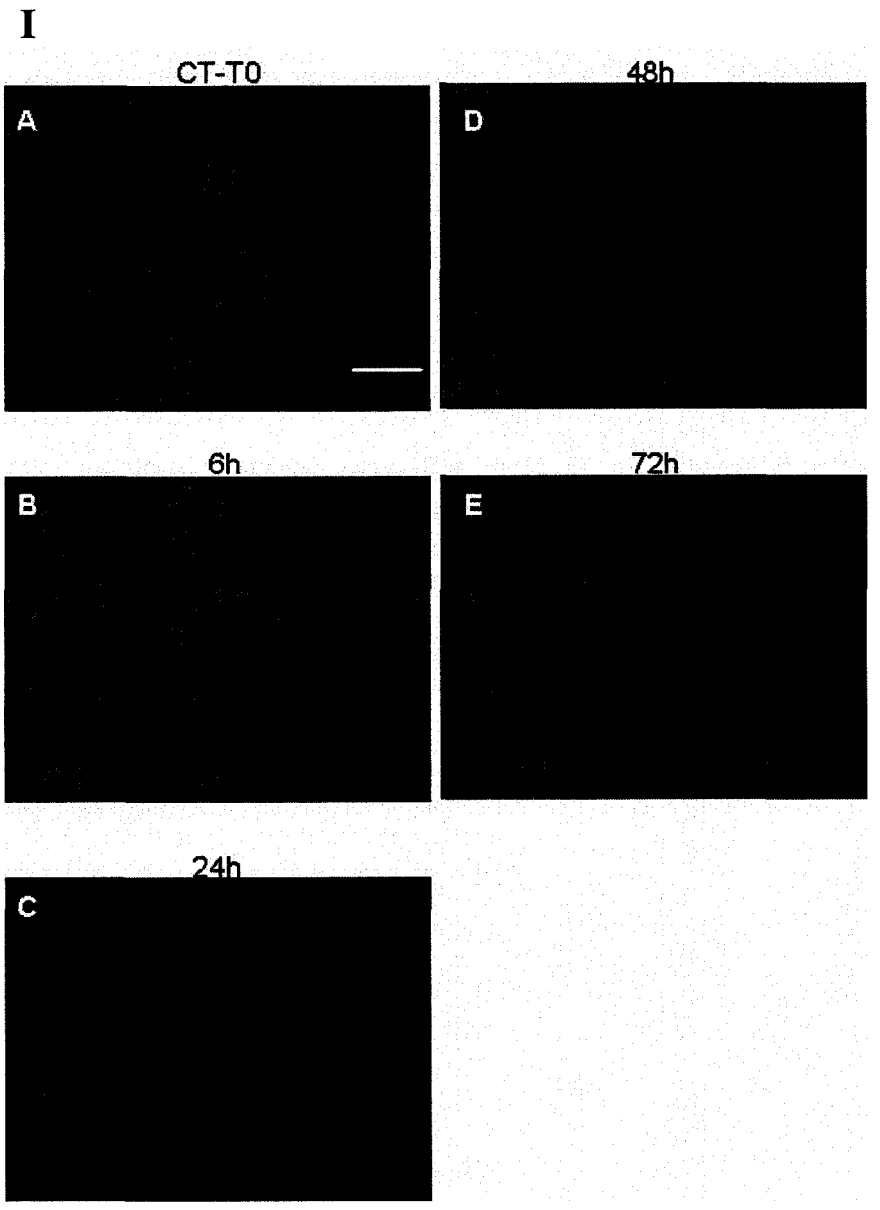


Figure 3.9I. Effect of BMP-2 on C2C12 commitment as measured by myogenin immunofluorescence. Cells were cultured under osteoblastic commitment conditions (300ng/ml of BMP-2) for the specified times and immediately fixed for detection with anti-myogenin (M-225) antibody as described in Materials and Methods, section 2.6.1. The micrographs were taken under similar conditions (duplicates were performed) in order to allow for intensity comparison. A low level of signal was observed (background) when the secondary antibody alone was used as a control. The line represents 25μm.

II

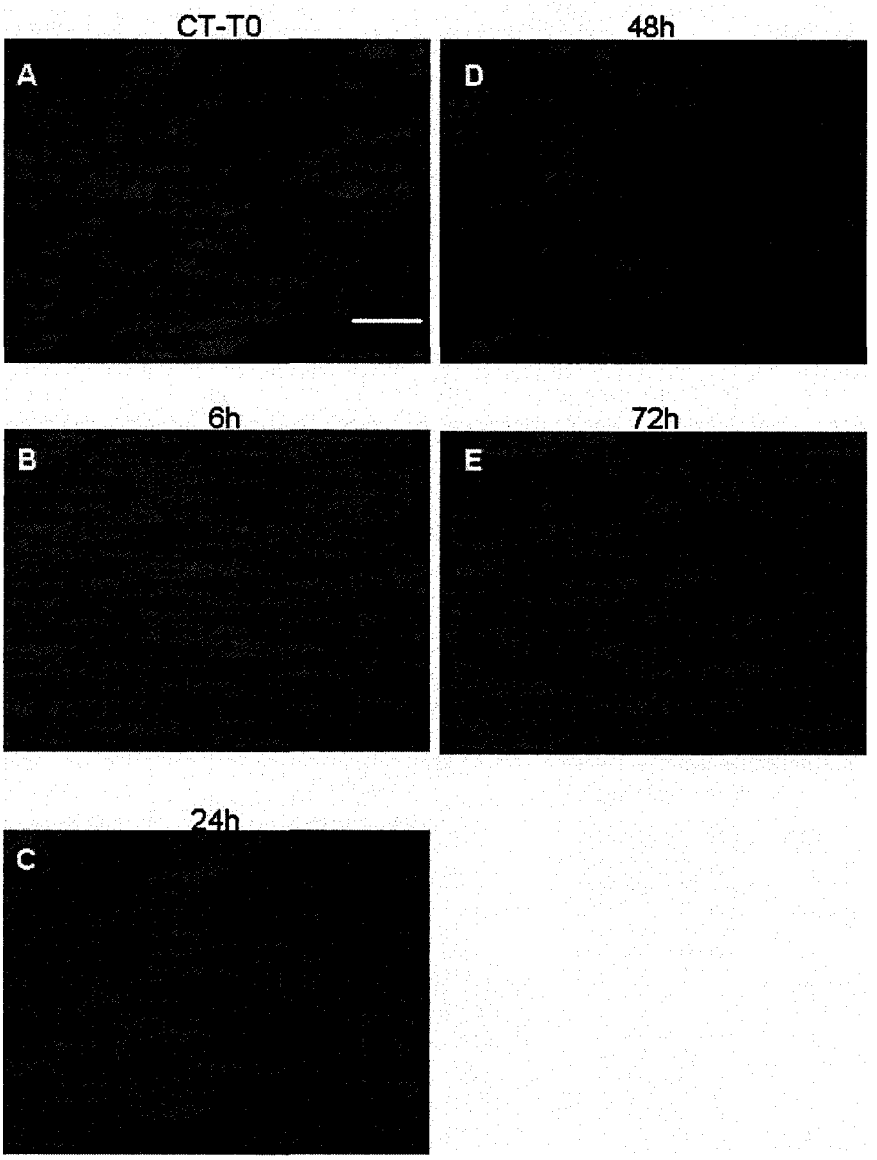


Figure 3.9II Effect of BMP-2 withdrawal on C2C12 commitment as measured by myogenin immunofluorescence. Cells were cultured under osteoblastic commitment conditions (300ng/ml of BMP-2) for the specified times, the medium was changed to 5% FBS without BMP-2, and then the cells were left in culture until completing a total culture period of 6d. Cells were fixed for detection with anti-myogenin (M-225) antibody as described in Materials and Methods, section 2.6.1 The micrographs were taken under similar conditions (duplicates were performed) in order to allow for intensity comparison. The line represents 25μm.

expression (Fig. 3.9 IIB), but exposures of 24, 48 or 72h (Fig. 3.9II C-E) strongly reduce the expression of myogenin as well as the number of spindle-shaped cells and syncytia.

When comparing the results from the ALP (section 3.2.1) and myogenin (section 3.2.2) assays it is clear that 24 to 48h exposure to BMP-2 leads to a large increase of ALP activity and modification of the sublocalization of the myogenin, with myogenin loosing its strong perinuclear location in a large number of progenitor cells. The typical multinucleated syncytia formed by myocyte fusion are present in confluent C2C12 populations cultured under myoblastic conditions (5% FBS, no BMP-2). The morphology of the most differentiated myoblast progenitors decrease in number as the duration of exposure to BMP-2 increases. Therefore the duration of the exposure to BMP-2 impacts on the distribution and the expression of myogenin. However myogenin is still expressed at more than background level after 72h exposure to the morphogen and a total of 6 days in culture (Fig 3.9IIE) ALP activity and myogenin immunofluorescence (Figure 3.8, 3.9) are complementary and suggest that if transdifferentiation is completed, maturation of the respective phenotypes is not accomplished by day 6. The maintenance of the BMP-2 signal seems not to be required to maintain high ALP activity but seems to be necessary to maintain the decline of myogenin during maturation. An alternative explanation is that different pathways contribute to the transdifferentiation of the C2C12 progenitors. We will return to this hypothesis later in the thesis.

3.3 The Smads Pathway in C2C12 Transdifferentiation

Smads proteins are widely expressed throughout development and are responsible for fate acquisition and/or the differentiation of osteoblasts (BMP-2/Smads-1, 5, 8) and

myoblasts (TGF-β/Smad-2). The fate and location of Smads during commitment/differentiation was investigated by immunofluorescence and WB. The level of unphosphorylated Smads was too low to be detected by WB and was when possible detected using immunofluorescence.

3.3.1 Smads Expression

3.3.1.1 Subcellular Localization of Unphosphorylated Smads-1, 5, 8

Unphosphorylated R-Smads reside in the cytoplasm, when phosphorylated they bind Smad-4 and translocate to the nucleus (Massague *et al.*, 1998). Unphosphorylated Smads-1, 5, 8 are located in the cytoplasm (Fig 3.10) at day 3, an expected result since it is only upon phosphorylation that they bind to Smad-4 and are transferred to the nucleus. Sublocalization did not change at day 6.

3.3.1.2 Subcellular Localization and Antibody Specificity of Phosphorylated (pSmads)-1, 5, 8

Preliminary experiments using anti-pSamds-1, 5, 8 (9511) and anti-Smad-8 (R-64) antibodies suggested that pSmads were always present (in the cells nuclei) even in untreated cultures. Two possibilities were raised: First that the antibody was labeling a perinuclear or nuclear protein in addition to the pSmads and second that one (or more) pSmad is constitutively expressed. Experiments were designed to test these hypotheses.

3.3.1.2.1 pSmads Antibodies Specificity: Is Labelling Truly Nuclear?

Confocal microscopy with successive z- sectioning was employed to eliminate the possibility that the antibody was tagging a nuclear envelope protein non-specifically. For this

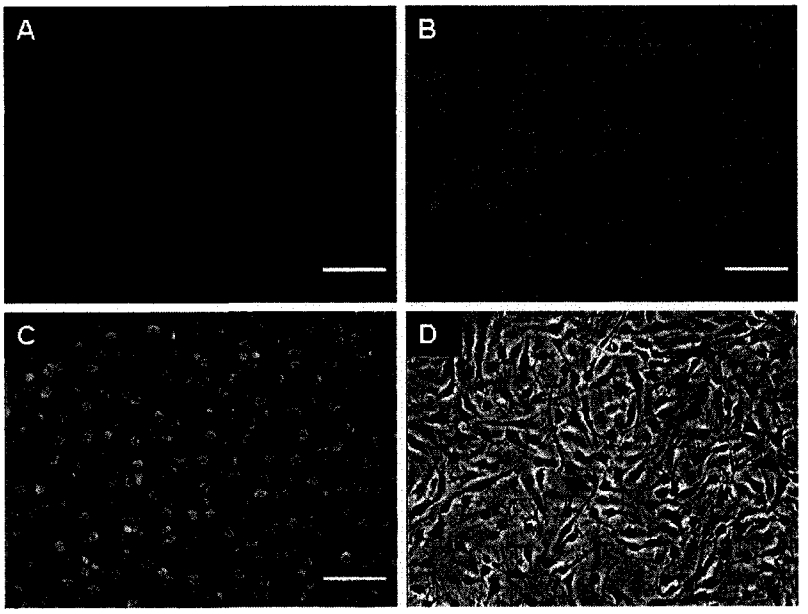


Figure 3.10. Sublocalization of Smads-1, 5, 8 under osteoblastic conditions-long term. C2C12 were cultured under osteoblastic and myoblastic conditions for 3 and 6 days. Samples were prepared for microscopy as indicated in Materials and Methods section 2.6. The anti-Smads-1, 5, 8 antibody N-18, specific for unphosphorylated Smads-1, 5 and 8 (Santa Cruz) was used. (A) DAPI nuclear staining; (B) immunolabeling of the protein(s); (C) Superposition of (A) and (B) demonstrating lack of co-localization (no yellow) at the nuclear level; (D) Phase contrast micrograph. The same results (not shown) were obtained at 3 and 6 days under all conditions. Duplicates were performed. The bars represent 50μm.

purpose, images were taken on the same cells at different heights (z-sections). If there was a substantial nuclear surface labelling, we would expect to see an empty nucleus with a surrounding ring indicating a membrane or perinuclear localization.

The localization of pSmads-1, 5, 8 is clearly nuclear since all the successive z-sections of confocal microscopy showed a solid nuclear signal (Fig 3.11) excluding the possibility that the antibody would label the nuclear envelope. In addition, it showed that the phosphorylated Smads were not present in what seem intranuclear organelles or other structures shown as dark areas in the nuclei. This result was confirmed by confocal microscopy with a second antibody, R-64 (Santa Cruz) that detects both Smad-8 and pSmad-8 (Fig 3.16). In this case, the cells plasma membrane was not labelled with the anti-wheat germ agglutinin antibody as in Fig. 3.11. Results similar to those in figure 3.11 were observed. The confocal experiments confirmed the nuclear localization of pSmads. In some sections, the anti-pSmad-1, 5, 8 antibody 9511 also decorated the cytoplasm of a few cells. This antibody may be detecting pSmads sequestered in the cytoplasm by I-Smads 6 and 7 that were extremely well expressed in our cell model and/or pSmads in transit to the nucleus (Cell Signalling, personal communication).

3.3.1.2.2 pSmads Antibodies Specificity: Is The Protein in Nuclear-Enriched Fractions?

R-Smads are expected to be mostly nuclear when phosphorylated. Cell fractionation and WB analysis were used to test this hypothesis. The enriched nuclear fraction shows higher level of pSamds-1, 5, 8 when compared to the cytoplasmic fraction (Fig 3.12) confirming the nuclear location of the proteins. The cytoplasmic fraction contained pSamds-1, 5, 8. This may be due to the release of these proteins from the nucleus during the cell

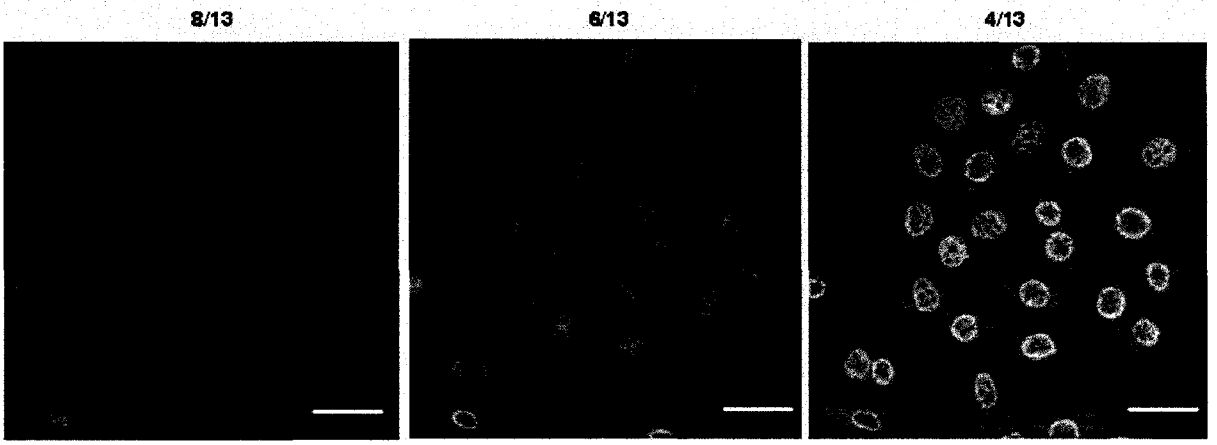


Figure 3.11. Confocal microscopy of pSmad-1, 5, 8 under myoblastic conditions. C2C12 cells were cultured under myoblastic conditions for 3 days. pSmads-1, 5, or 8 were detected by immunolabelling with anti-pSmad-1,5,8 antibody 9511 and anti-wheat germ agglutinin antibody W849 as per Materials and Methods section 2.6.1. Confocal microscopy was performed as per Materials and Methods section 2.6.3. Rhodamine-conjugated agglutinin binds membrane glycosylated proteins and fluoresces in red, while the green fluorescence represents the pSmad-1, 5, 8. The bar represents 50 μm. The numbers indicate the number of the z-sections out of a total of 13. The seconday antibody control yielded a very low signal.

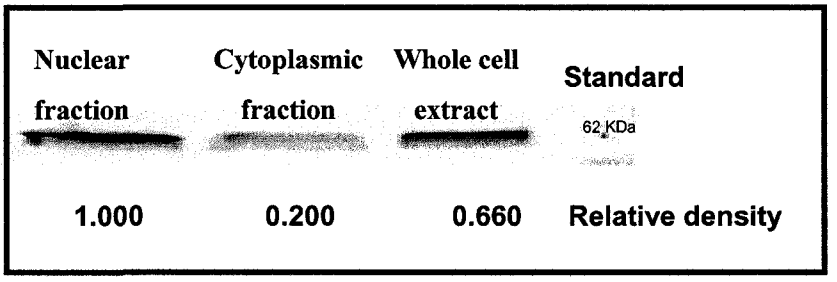


Figure 3.12 Nuclear extraction profile of pSmads-1, 5, 8. Cells were cultured for 3 days under osteogenic conditions. Extractions and WB of the enriched nuclear fraction, cytoplasmic fraction and whole cell extraction (RIPA) were performed as per Materials and Methods sections 2.4.2 and 2.5.4 with anti-pSmad-1, 5, 8 antibody 9511. Band density estimated as per Materials and Methods section 2.5.5. Experiments were performed in duplicate. The proteins were processed on the same membrane. Complete blot can be seen in appendix A.

fractionation and/or the presence of pSmads in the cytoplasm. The specificity of the pSmad-1, 5, 8 antibody was also successfully tested by starvation (appendix B). Confocal microscopy produced images that further proved the specificity of the anti-pSmad-8 antibody (Fig 3.16 I).

3.4. The Fate of Smads during Osteoblastic Commitment/Differentiation

When BMP-2 evokes the Smads pathway, ligand-receptor complexes phosphorylate Smad proteins thus activating them. Changes in Smads sublocalization were monitored by immunofluorescence after exposure to BMP-2 for short times (from 10 minutes to 4 hours) and for longer terms (3 and 6 days) under control (myoblastic) and osteoblastic conditions. Although experiments were performed with antibodies against Smad-4, Smad-6, Smad-7, and pSmad-1 (Materials and Methods, Table 2.1), most of the experiments reported below investigated pSmad- 1, 5, 8 and Smad-8/pSmad-8 that will be the focus of this section of the thesis.

3.4.1 Fate of pSmads, and I-Smads under Short Term Commitment Conditions

Antibody 9511 recognizes phosphorylated Smads-1, 5 and/or 8 (pSmad-1, 5, 8) (Fig 3.13). At 4 hours the proteins are almost completely nuclear (blue arrows) both in untreated cells (I) and cells exposed to BMP-2 (II) with very little staining found in the cytoplasm. The same sublocalization was constantly found throughout all the time points (from 10 min to 4 hours) whether the cells were treated or not with BMP-2 in 5%-FBS supplemented medium. Antibody R-6 recognizes unphosphorylated and phosphorylated Smad-8 (Fig 3.14). As with the pSmad-1, 5, 8 results, at 4 hours most protein was nuclear. The same result was found

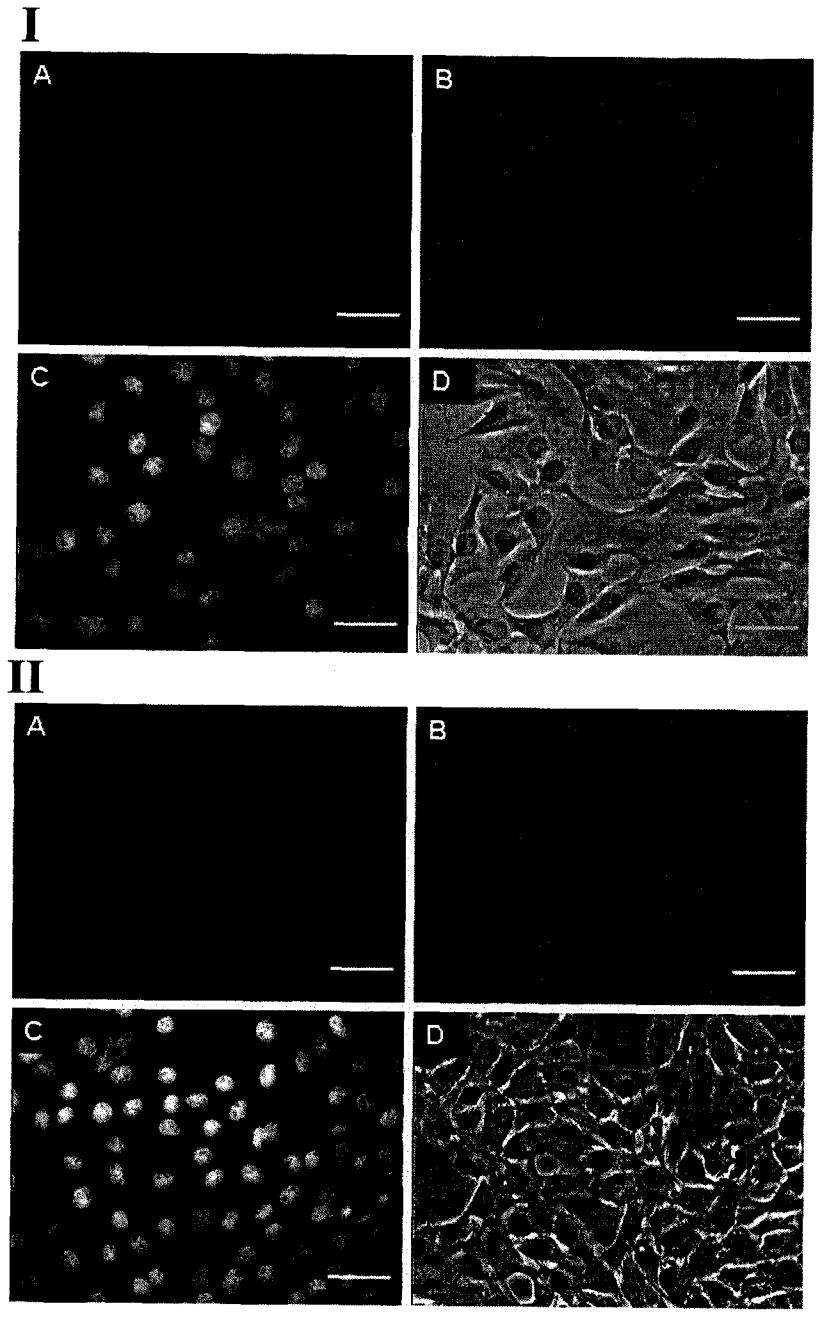


Figure 3.13. Cellular sublocalization of pSmads-1, 5, 8 under commitment conditions. C2C12 were cultured under commitment conditions for 4 hours (in duplicate, in medium with 5% FBS) with or without BMP-2. Samples were prepared for microscopy as indicated in Materials and Methods section 2.6. The anti-pSmads-1, 5, 8 antibody 9511 (Cell Signaling) was used. **(I)** Images of pSmads-1, 5, 8 at 4 hours without treatment with BMP-2. **(II)** Images of pSmads-1, 5, 8 at 4 hours after treatment with 300ng/ml of BMP-2. Panels A, B, C and D depict the micrographs as in figure 3.10. Duplicates were performed. Some pictures were enhanced to facilitate their interpretation; therefore, quantification of the protein is not feasable. The arrows point at nuclei containing the phosphorylated protein (s). The bars represent 25 μm.

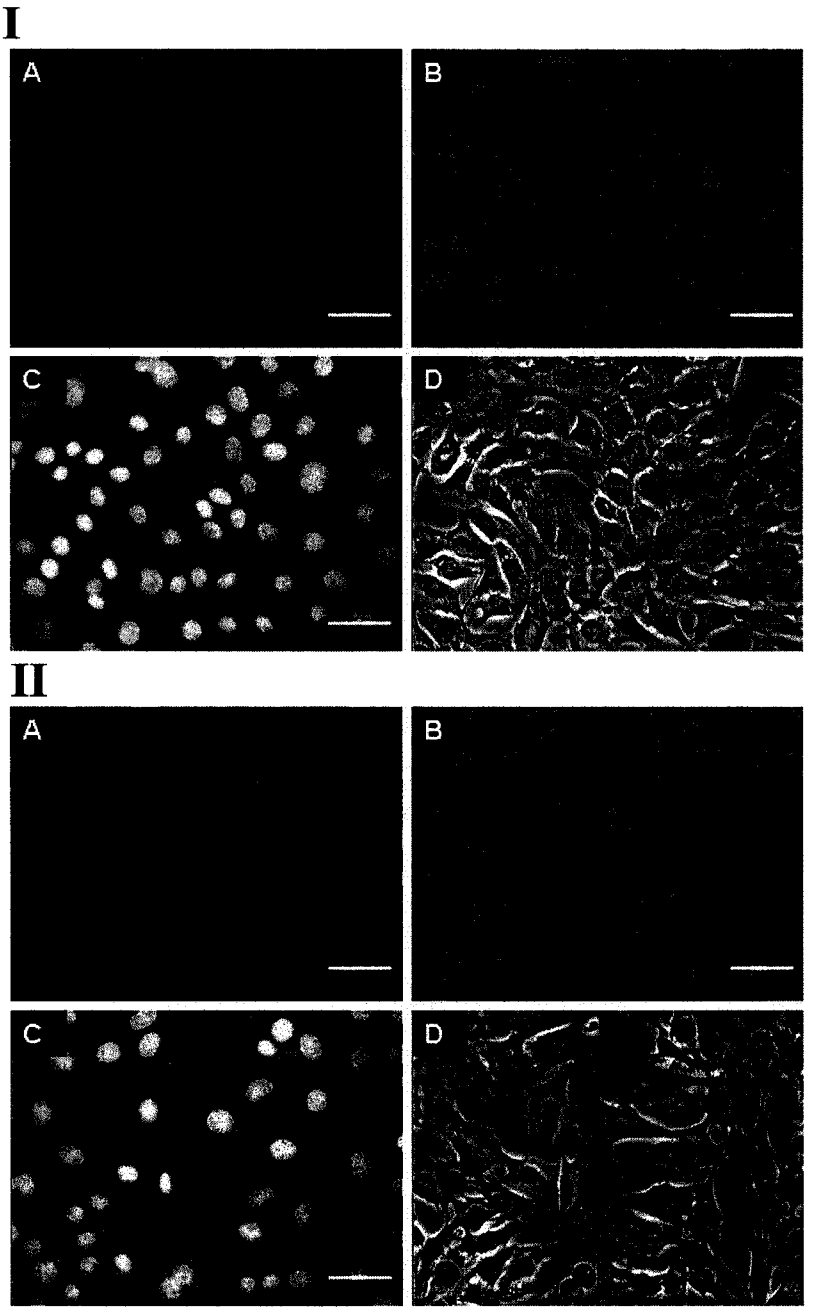


Figure 3.14. Cellular sublocalization of Smad-8 and pSmad-8 under commitment conditions. C2C12 were cultured under committing conditions for 4 hours in medium with 5% FBS with or without BMP-2. Samples were prepared for microscopy as indicated in Materials and Methods section 2.6. The anti-Smad-8 dual antibody R-64 (Santa Cruz) was used. **(I)** Images of (p)Smad-8 at 4 hours without treatment with BMP-2. **(II)** Images of (p)Smad-8 at 4 hours after treatment with 300ng/ml of BMP-2. Panels A, B, C and D depict the micrographs as in figure 3.10. Duplicates were performed. Some pictures were enhanced to facilitate their interpretation; therefore, quantification of the protein is not feasable. The bars represent 25 μm..

regardless of the time (10 min to 4 hours) or the treatment; however more stain was cytoplasmic as expected for an antibody that recognizes both forms of the protein. The fate of the inhibitory Smad-7 was monitored using the antibody H-79 (Santa Cruz). Smad-7 was found both at the nuclear and cytoplasmic level under both osteoblastic and myoblastic conditions through all time-points in the short term kinetics study (appendix B). Similar results were seen for Smad-6 visualized with the antibody H-150 (Santa Cruz). The presence of Smad-6 in the cytoplasm but also in the nucleus has been previously reported (Bai and Cao 2002). pSmad-1 visualized with the antibody sc-12353 (Santa Cruz) showed some nucleus-cytoplasm shuttling; the protein was found in both nuclear and cytoplasmic sublocalizations (appendix B).

Taken together these results indicate that at early times, the pSmads proteins are found mostly in the nucleus. This suggests that an unknown factor is activating the cell signaling causing Smads phosphorylation and shuttling to the nucleus prior to the addition of BMP-2. The shuttling may be rapid since cytoplasmic unphosphorylated Smads are not detected often or at high levels.

3.4.2 Fate of Smads under Long Term Commitment Conditions

Experiments similar to those in section 3.4.1 were performed by monitoring both immunofluorescence and WB at 3 and 6 days, the time frame in which commitment to transdifferentiation occurs. Again, only representative data are shown.

3.4.2.1 Immunofluorescence Results

At day 3 pSmads-1, 5, 8 are located in the nucleus of C2C12 cells (Fig 3.15 I), pSmad-8 is

located mainly in the nucleus (Fig 3.15 II) and Smad-4 is distributed evenly in the cytoplasm and the nuclei of cells (appendix B). Similar results were found under osteoblastic conditions at 3 days and under osteoblastic and myoblastic conditions at day 6 (data not shown).

These results support the short term ones, where Smads when phosphorylated were located in the nucleus almost at all times and under a wide array of conditions. The cytoplasmic location of some Smad-8 and Smad-4 are consistent with the known specificities of the antibodies. As previously shown with the dual anti Smad-8 antibody (recognizing Smad-8 and pSmad-8), some of the signal is cytoplasmic.

As done previously (Fig 3.11) confocal microscopy of whole cells and z-sections of BMP-2 treated cells (Fig 3.16 I and II) were performed to confirm that pSmad-8 was truly nuclear. pSmad-8 is clearly seen present inside (yellow arrow) the nucleus. Interestingly nuclei without pSmad-8 (blue arrows) are found and their distribution depends upon the focus indicative of a second cell layer. As with previous experiments, this may indicate heterogeneity in response to BMP-2.

The fate of pSmad-1, 5, 8, (p)Smad-8, Smad-4, Smad-7, and Smad-6 were monitored by WB analysis with the same antibodies used above in the immunofluorescence studies (Table 2.1). All inferences depend upon duplicate experiments and are thus qualitative. As will be briefly described below, all Smads investigated are detectable regardless of the culture conditions and time points. The level of the Smads under pluripotent conditions (10% FBS without BMP-2) was monitored as well.

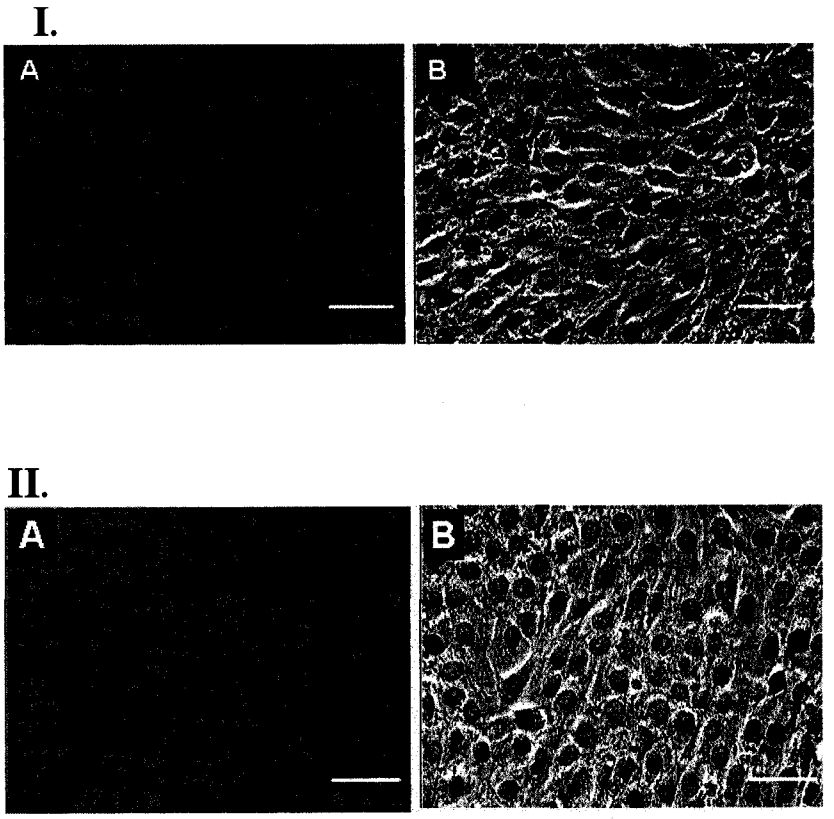


Figure 3.15. Cellular sublocalization of pSmads-1, 5, 8 and Smad-8 under long term growth conditions. C2C12 were cultured for 3 days in medium with 5% FBS without BMP-2. Samples were prepared for microscopy as indicated in Materials and Methods section 2.6. The anti-pSmad-1, 5, 8 antibody 9511(Cell Signaling) and the anti-Smad-8 dual antibody R-64 (Santa Cruz) were used. **(I)** Images of pSmad-1, 5, 8 at 3 days without treatment with BMP-2. **(II)** Images of Smad-8 at 3 days without treatment with BMP-2. (A) immunolabeling of the protein(s); (B) Phase contrast micrograph. Some pictures were enhanced to facilitate their interpretation; therefore, quantification of the protein is not feasable. Duplicates were performed. The bars represent 50μm..

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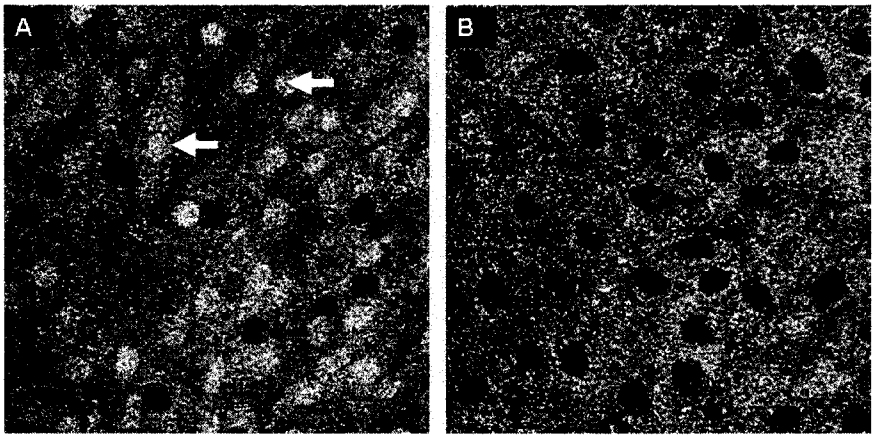

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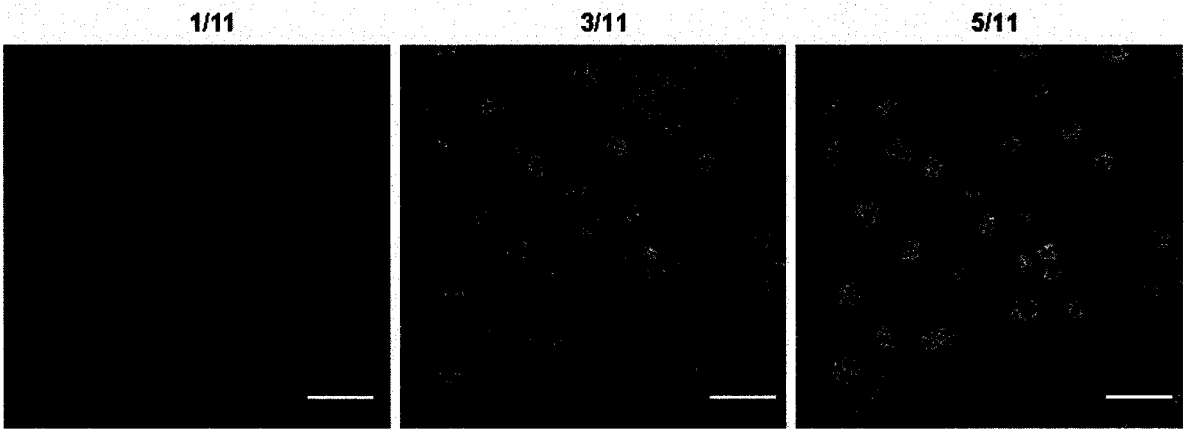


Figure 3.16. Confocal microscopy of Smad-8 and pSmad-8 under osteoblastic conditions.

C2C12 cells were cultured under osteoblastic conditions for 6 days. Smad-8 and pSmad-8 were detected by immunolabelling with dual antibody R-64 (Santa Cruz) as per Materials and Methods section 2.6.1. Confocal microscopy was performed as per Materials and Methods section 2.6.3. (IA) Section z 14 of 21 of confocal microscopy. (IB) whole cell image focusing on a lower layer showing mainly unlabelled nuclei. The yellow arrows point at empty nuclei while the blue arrows point at nuclei containing the phosphorylated protein. II z sections showing the nuclear localization of the protein. The bars represent 25μm..

3.4.2.2 Western Blot Results

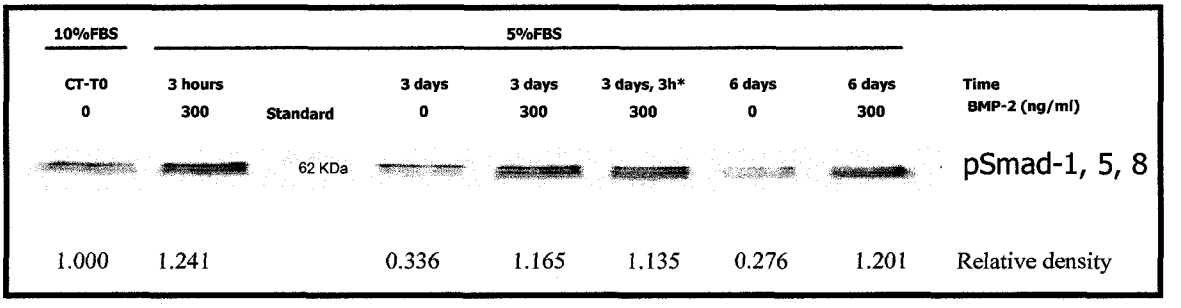
The antibody 9511 recognizes the phosphorylated forms of Smads-1, 5, 8. Based upon the apparent molecular weight, the observed bands (Fig 3.17) correspond to phosphorylated species (Santa Cruz, MW 60KDa). The proteins are detected at all time points including the pluripotent

stage (CT-T0 10% FBS). Cells not exposed to BMP-2 did generate a weaker band meaning that Smads are being expressed and phosphorylated under reduced serum conditions in the absence of BMP-2. Therefore, Smads-1, 5 and/or 8 seem be expressed in C2C12 cells and phosphorylated even under pluripotent conditions and in untreated controls. This is a novel observation. Exposure to BMP-2 noticeably increased expression at 3 hours, indicating that BMP-2 receptors that phosphorylate Smads were functional. The sustained BMP-2 signal (beyond 3 hours up to 6 days) did not result in a larger increase of pSmads (as expressed on a per protein basis) over time. This suggests that a stronger response is not generated by the extra BMP-2 treatment.

Smad-8 and /or pSmad-8 (expected MW 52 KDa) were detected as a single faint band under all conditions. Poor signal intensity was observed and may be attributed to the antibody response on WB since the very same antibody exhibits very good staining properties in immunofluorescence experiments. Under the different culture conditions, the Smad-8 protein detected by WB exhibited <2-fold changes in band intensity which may not be significant (data not shown). More replicates are needed to draw conclusions. The co-Smad-4 is well expressed through all time points under the different treatments as are also the I Smad-6 and Smad-7 (data not shown).

These results confirm what was seen with the immunofluorescence studies; namely, that pSmads of interest are expressed under all conditions at all times. As stated previously

A



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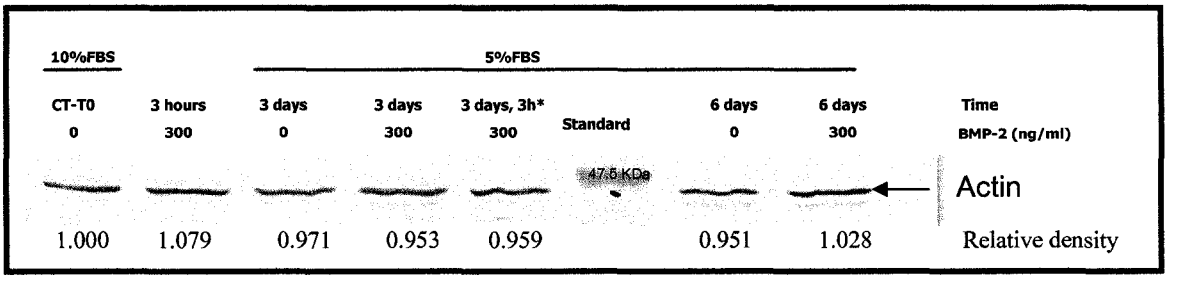

Figure 3.17. pSmads-1, 5, 8 expression under different culture conditions. C2C12 cells were cultured under the specified conditions. Extracts were prepared and 30ug of protein were loaded, separated by electrophoresis, transferred onto nitrocellulose membranes and detected with (A) the anti-pSmad-1,5,8 antibody 9511 (Santa Cruz) or (B) the anti-actin antibody I-19 (Santa Cruz) (actin is a house keeping gene used as loading control). Densitometry was performed as specified in Materials and Methods section 2.5.5. The standard lane in A shows the 60KDa band. Duplicates were performed. CT-T0 cells were extracted after overnight settling in subculturing medium (10% FBS without BMP-2). The other treatments were started after cell settling by changing to commitment medium (5%-FBS with or without 300ng/ml of BMP-2) and incubated for the times indicated prior to processing. In the case of cells under 5% FBS treatment 3 days, 3h* with 300ng/ml of BMP-2, at 3 days the medium and BMP-2 were changed for fresh ones. Three hours later, the cells were extracted as per Materials and Methods.The proteins were processed on the same blots. Complete blots can be seen in appendix A.

this suggests that signaling via the Smads pathway occured in the absence of BMP-2 which is an important unexpected finding.

3.5 Smads are Expressed and Phosphorylated in Pluripotent Cultures

Experiments were done to investigate the sublocalization and expression of some pSmads at the pluripotent stage (subculturing with 10% FBS) preceding the "myogenic and osteogenic culture" conditions to determine the basal state expression level and localization of these proteins. The sub localization was examined by immunofluorescence. Under proliferative conditions, pSmads-1, 5, 8 (Fig 3.18 I) are localized mainly to the nucleus as can be seen by the superposition of the fluorescence and DAPI signals (panel C) which results in an orange and/or yellow color in the overlap. pSmads appear to be excluded from what may be nuclear organelles or condensed chromatin (panel E arrows). Very little or no signal is cytoplasmic (white arrow). Finding a label was unexpected since the dogma is that it is only upon BMP-2 addition that the Smads become phosphorylated. This raised questions as to the identity of the signals promoting the phosphorylation in the absence of BMP-2 (analyses of FBS did not reveal the presence of BMP-2). Constitutive activation is also improbable since we demonstrated Smads shuttling and a lower signal under starvation (appendix B).

The anti-Smad-8 antibody decoration is mainly located in the nucleus in cells cultured under pluripotency conditions (Fig 3.18 II); one can conclude that most Smad-8 is found in nuclei in the phosphorylated form, although some unphosphorylated protein pSmad in transit or cytoplasm-sequestered pSmad-8 is seen in the cytoplasm (white arrow). The high rate of Smad-8 in phosphorylated form under proliferation conditions was unexpected. Smad-7 was located mainly in the cytoplasm and I Smad-6 was located mainly in the nucleus although

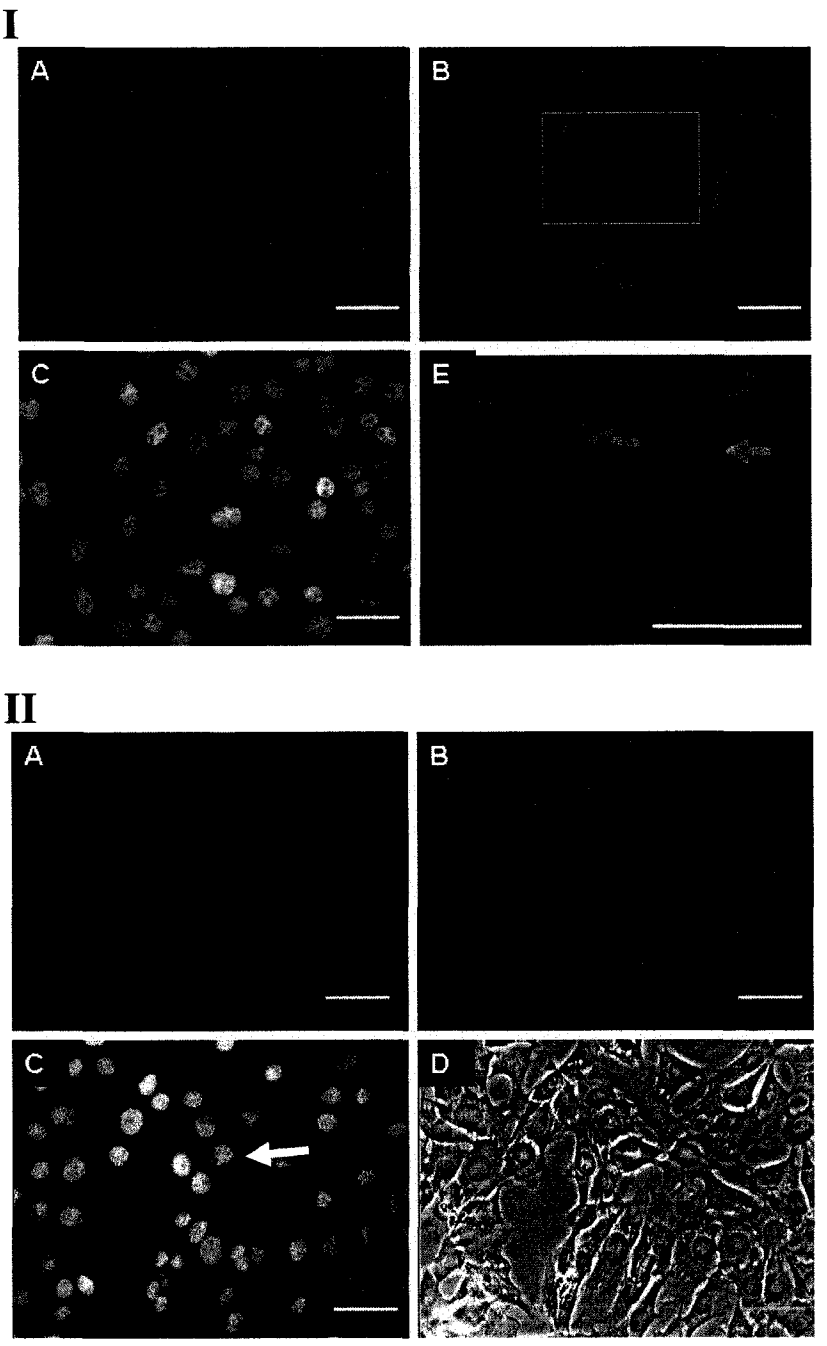

Figure 3.18. Localization of pSmads-1, 5, 8 and Smad-8 under proliferative conditions. C2C12 were cultured under proliferative conditions (10% FBS) for 16h. Samples were prepared for microscopy as indicated in Materials and Methods section 2.6. (I)The anti-pSmads-1, 5, 8 antibody 9511 specific for phosphorylated Smads-1, 5, 8 (Cell Signaling). The blue arrows point at the heterogeneous distribution of the protein in the nucleoplasm. (II) The anti-Smad-8 dual antibody R-64 recognizing unphosphorylated and phosphorylated Smad-8 (Santa Cruz) was used. The white arrow shows unphosphorylated Smad-8 present outside the nucleus. Panels A, B, C, and D as in graph 3.13; (E) amplification of box located in IB. Duplicates were performed. The bars represent 25μm.

some protein was also localized to the cytoplasm (appendix B).

The results clearly show that the Smads pathway is already active in the uncommitted C2C12 line prior to activation by a morphogen such as BMP-2. When cells were starved by culturing in 0.5% FBS for 24 h and then switched for 15 min in 10% FBS, the intensity of nuclear staining with anti-pSmad-1, 5, 8 antibody 9511 (Cell Signaling) increased perhaps indicating a phosphorylating role for a growth factor contained in FBS. Therefore, it was hypothesized that a factor present in the FBS may be activating the Smads pathway and/or another pathway that may also operate in transdifferentiation. With the goal of finding some information in the matter, experiments were performed as indicated below.

3.6 TGF-β and p38 MAPK Pathways: Potential Involvement in C2C12 Transdifferentiation

Data presented in this thesis including the expression of myogenin after 6 day exposure to 300ng/ml of BMP-2, and the continuous presence of phosphorylated Smads especially in the proliferative phenotype, prompted an investigation of whether more than one pathway was contributing to the transdifferentiation and/or the maturation of the osteoblastic lineage.

ALP expression is known to be basically under the control of 2 pathways: the p38 MAPK (Nohe *et al.*, 2002) and the Smads pathways (Yamamoto *et al.*, 1997). TGF–β1 is a growth factor capable of activating the Smads-2, 3 via ALK5 as well as the BMP/Smads-1, 5 and/or 8 pathway via ALK1 (Larsson and Karlsson, 2005) and ALK4 (Inman *et al.*, 2002). TGF–β1 is secreted by C2C12 cells as an autocrine/paracrine growth factor (Yoshiko *et al.*, 2002) and is also present in FBS (FBS supplier analysis). The TGF–β1 molecule through activation of the ALK 1 and ALK 5 elicited pathways may influence the phosphorylation status of the BMP pathway's Smads and TGF-β pathway's Smads (Smads-2 and 3) and thus

contribute to the transdifferentiation process. TGF-β1 can also activate p38 MAPK independently from Smad transcription (Derynck and Zhang, 2003).

The contributions of the TGF-β1 and p38 MAPK pathways in transdifferentiation were assessed using a pharmacological approach: SB202190 known to directly inhibit p38 MAPK was used as well as SB431542 that inhibits TGF-β signaling by blocking the protein kinase activity of ALK-4 (type I receptor for Activin), ALK-5 (type I receptor for TGF-β, ten Dijke *et al.*, 2003) and ALK-7 (type I receptor for nodal a member of the TGF–β superfamily), without affecting BMP signaling.

Table 3.2 reports the treatments used to evaluate the effect of TGF-β (through ALK4) and the p38 MAPK pathways in the transdifferentiation of C2C12 cells and their effects in the cell population. Addition of BMP-2 increases cell proliferation (compare D to B to A). The addition of SB431542 diminishes the BMP-2 induced increase but not below the levels in the untreated population while the addition of SB202190 blocks proliferation and may even reduce growth to below control values. The cells that responded to BMP-2 exposure in terms of proliferation have not been identified. The population number changes were taken into account in the calculations.

3.6.1. Assessing the Influence of the TGF-β and p38 MAPK Contributions by Measurements of ALP Activity During Commitment

3.6.1.1 ALP Activity in Cell Lysates

The ALP activity was monitored under commitment conditions and in the presence or not of BMP-2 at two doses, SB202190 and/or SB431542 according to the schedule in table 3.2. Since transdifferentiation occurs in patches (Fig. 3.3B and 3.4) ALP activity per individual cell may vary. The values presented here are calculated over the cell population.

TREATMENT	BMP-2 ng/ml	SB431542 µM	SB202190 µM	Population size
A	0	0	0	***2619.2***
B	50	0	0	***3044.7***
C	50	10	0	***2621.7***
D	300	0	0	***4106.7***
E	300	10	0	***3465.2***
F	50	0	10	***2014.2***
G	300	0	10	***1930.7***
H	50	10	10	***1843.7***
I	300	10	10	***1815.7***

Table 3.2. Experimental approach used to study the effect of TGF-β and p38 MAPK pathways on C2C12 cell commitment. Cells were cultured in 5% FBS under the specified conditions for 6 days, and then ALP activity with pNP substrate was quantified as per Materials and Methods, section 2.7.1. The activity of ALP was also assessed by cellular activity on BCIP (staining) and back-extraction of the coloured BCIP/NBT complex as per section 2.7.2. The last column reports the estimation of the cell population at 6 days of culture as described in Materials and Methods. The reported fluorescent relative units are directly related to the number of cells in the different treatments. The population size was estimated using the methodology described in section 2.3.1 (Population Size Estimation) and is reported as relative fluorescence units.

When BMP-2 was present at 50ng/ml ALP activity increased. ALP activity declined with the addition of SB431542 or SB202190; however, these observed changes were borderline in terms of significance. When BMP-2 was present at 300ng/ml a large increase in ALP activity was observed (Fig 3.19). The addition of either inhibitor reduced the ALP activity to control levels. The presence of both inhibitors did not reduce ALP activity much further. The values of A, E, G and I are significantly different from D (ANOVA, $p<0.05$).

These results suggest that TGF-β1 and p38 MAPK pathways share some commonality. Furthermore, the contribution of the Smad-1, 5, 8 pathway in increasing the activity of ALP may be modest or may result from cross signaling with the p38 MAPK and TGF-β pathways. Cross signaling is commonly observed among these pathways in different cell types (see Discussion).

3.6.1.2 In situ ALP Activity and Back-extraction of NBT/BCIP Substrate

As with previous experiments, a second method (back-extraction) was used to validate the above results. For cells exposed to 50ng/ml of BMP-2 alone and with the inhibitors (Fig 3.20 I) the changes observed were minor and not significant. In cells exposed to 300ng/ml of BMP-2 (Fig 3.20 I and II) the level of ALP was much increased and exposure to the TGF-β receptor inhibitor or the p38 MAPK inhibitor decreased ALP activity when compared to cells exposed only to BMP-2. Exposure to BMP-2 and both inhibitors in combination (treatment I) reduces the ALP activity level further suggesting an additive effect of the two inhibitors.

These results agree with the data presented in section 3.6.1.1 and figure 3.19. The addition of 300ng/ml BMP-2 increases ALP activity and the simultaneous addition of either

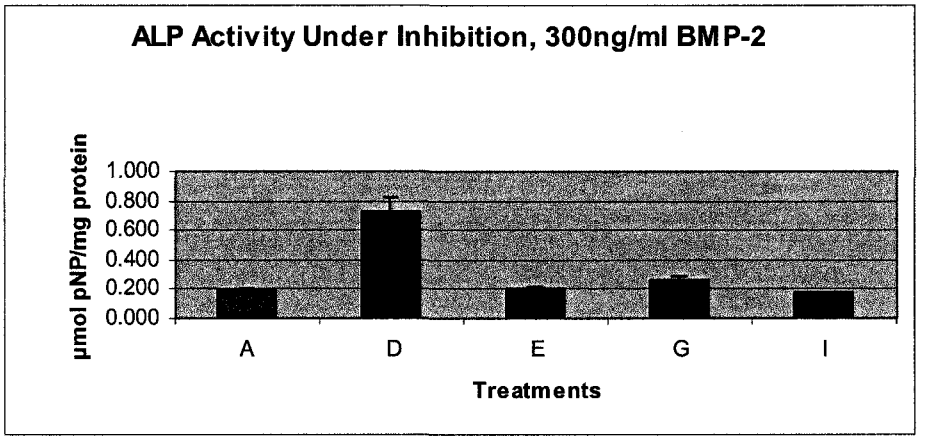

Figure 3.19. The contribution of the p38 MAPK and TGF-β pathways to transdifferentiation: measurements of ALP specific activity by pNP. ALP activity was measured after 6 days under culture commitment conditions and exposure to 300ng/ml BMP-2 and selective inhibitors of the p38 MAPK and TGF-β pathways. The treatments are described above in Table 3.2. ALP extraction and activity estimation were performed as described in Materials and Methods, section 2.7.1. Four replicates were performed.

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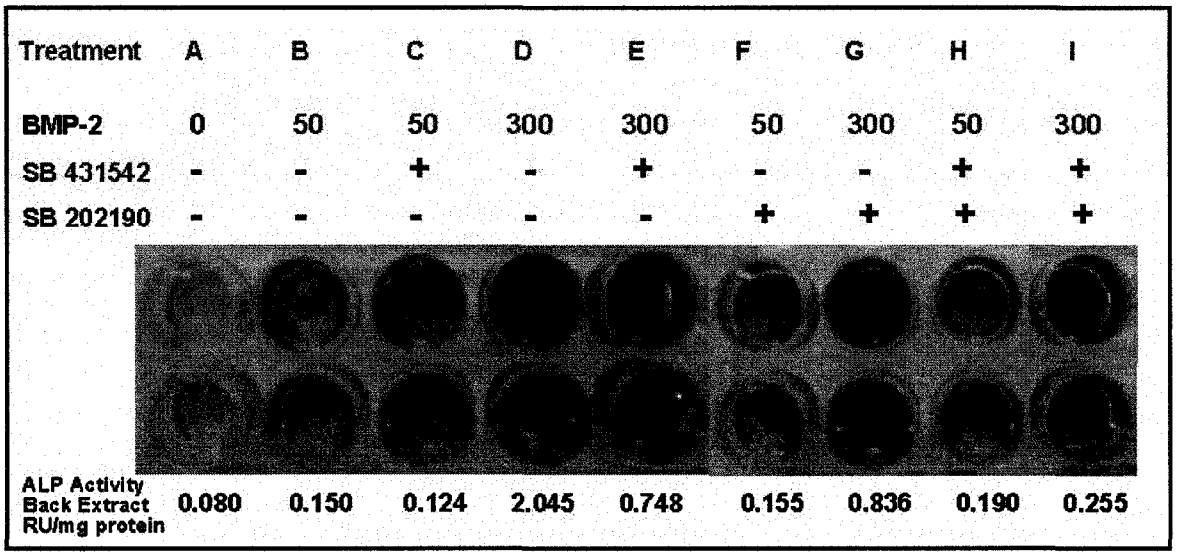

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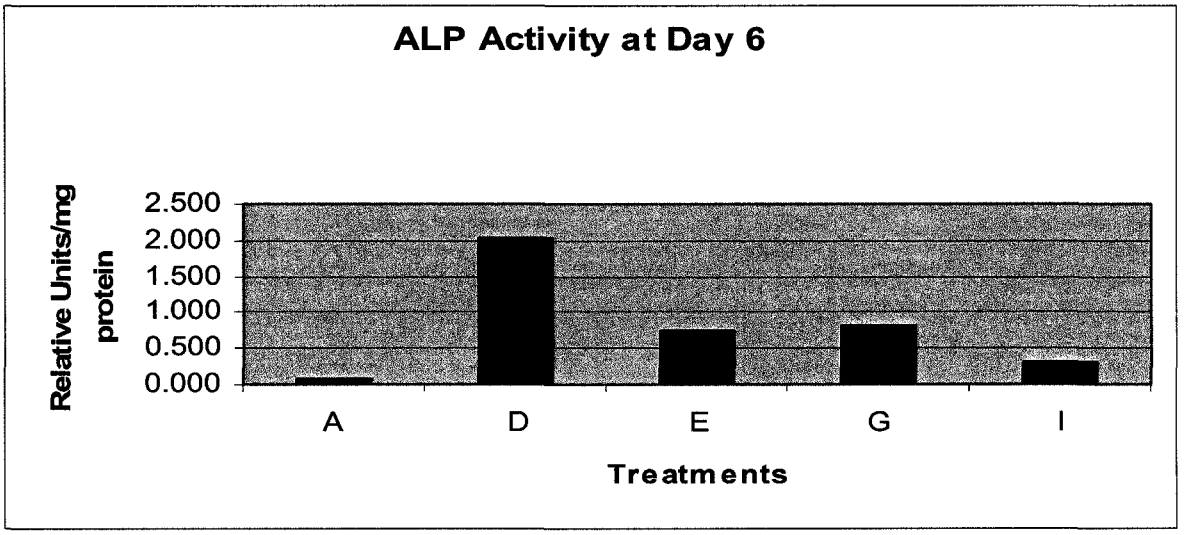

Figure 3.20. The contribution of the p38 MAPK and TGF-β pathways to transdifferentiation: measurements of ALP activity by staining and back-extraction. ALP activity was measured after 6 days under culture commitment conditions and exposure to 300ng/ml BMP-2 and selective inhibitors of the p38 MAPK and TGF-β pathways (Materials and Methods, section 2.7.2). The treatments are described above in Table 3.2. RU is relative units. (I) Activity as visualized by NBT/BCIP staining. (II) ALP activity for treatments at 300ng/ml of BMP-2. Experiments were done in duplicate; the average is shown.

inhibitor lowers ALP activity. A difference between the two results is observed when both inhibitors are added. In 3.6.1.1 no additive effect is seen, in 3.6.1.2 an additive effect is seen.

The differences between the results may relate to the different assay techniques used.

Chapter 4
Discussion

The potential use of embryonic stem cells as a therapeutic tool is highly controversial. Adult multipotent progenitor cells may thus represent an important alternative especially since they are easily acquired from the same person and no immunological rejection should be expected. The present research project attempted to better understand the commitment and early maturation of an adult multipotent progenitor cell to an osteogenic lineage under more physiological conditions. For this purpose, changes following treatment with BMP-2 were monitored without additional changes to selective media or transfection. BMP-2 has bone inductive properties, and as with all other BMPs, transmits its signal through the Smads pathway. BMPs and Smads are involved in the commitment of embryonic stem cells (ES) and adult progenitor cells to specific lineages and also in several types of cancer. Research devoted to the understanding of the mechanisms used by growth factors to direct crucial cellular activities such as proliferation, commitment, differentiation and death is clearly important for many fundamental reasons and for the implementation of potential therapies.

Summary of Results

The pluripotent precursors C2C12 were transdifferentiated to the osteoblastic lineage after exposure to BMP-2. The response to BMP-2 was dose and time dependent indicating the presence of functional BMP-2 and its receptors. At a low dose of BMP-2 (50ng/ml) an increase in ALP activity was observed without repression of the default myoblastic phenotype. At 300ng/ml, BMP-2 caused an increase in ALP activity and repression of the default myoblastic phenotype as illustrated by a progressive decrease in myogenin

expression. Even under these latter conditions, a small part of the cell population did not transdifferentiate suggesting that there is heterogeneity in the C2C12 population. As detected by WB analysis, myogenin was still weakly expressed at 6 days under BMP-2 treatment; this was confirmed in immunofluorescence by the occasional presence of spindle-shaped cells still strongly expressing the protein. The approximate timing of commitment to transdifferentiation was established for the first time by monitoring the potential for ALP activity stability upon BMP-2 withdrawal: Time dependent commitment to the osteoblastic lineage started at or before 6h exposure to 300ng/ml BMP-2 and continued over time. The number of committed cells and the simultaneous increase in ALP activity with time stabilized at 48-72h. What could be regarded as regression –as judged by ALP activity levels- in a 6-day trial yielded results where ALP activity seems to slightly decrease over time upon BMP-2 withdrawal.

The sublocalization of Smads under the different treatments was investigated, pSmads were unexpectedly well detected under all conditions, including subculturing and at all time-points; and localized (as expected) to the nucleus. The kinetics of pSmad-8 sublocalization that, to my knowledge, has not been studied in detail yet, also demonstrated constant presence of the protein accompanied by the expected nuclear localization. The fact that phosphorylated Smads are also found under proliferation conditions suggests that morphogens other than BMP-2 may contribute to the early maturation of C2C12 osteoblasts.

The contributions of TGF-β1 and the p38 pathway were assessed. Surprisingly, the contribution of TGF-β1 and the p38 pathway were as crucial in the process of early maturation as the BMP-2 elicited Smads pathway, at least under our cell culture conditions.

Validation of the Cell Model and Evolution of Differentiation Markers

C2C12 cells showed the typical morphology of skeleton muscle cell precursors or osteoblasts (depending on culture conditions) in agreement with previous results (Murray *et al.*, 1993; Katagiri *et al*, 1994; Lee *et al.*, 1999; Mermelstein *et al*, 2003; Meaeda *et al.*, 2004).When cultured in 5%-FBS without BMP-2, cells became oriented and then fused to form multinucleated syncitia (Katagiri *et al*, 1994. Burattini *et al.*, 2004) known as pre-myotubules. Although not reported in the literature, the formation of at least two layers of cells was observed during the later stages of the experimentation. Cells cultured in 5% FBS and 300ng/ml of BMP-2 transdifferentiated to the osteoblastic lineage showing the previously reported polygonal shape typical of the phenotype (Katagiri *et al.*, 1994; Gu *et al.*, 2004).

Alkaline phosphatase activity was monitored during transdifferentiation. This enzyme is widely accepted as a marker for differentiating osteoblasts both *in vivo* and *in vitro.*The tissue nonspecific or kidney/bone/liver (TNSALP) isoenzyme is the major isoform expressed in osteoblasts (Kim *et al.*, 2004). The exposure of C2C12 cells to BMP-2 triggered an increase in ALP activity that was dose and time dependent in agreement with other authors' observations (Katagiri *et al.*, 1994; Gallea *et al.*, 2001). At the same time the level of myogenin declined.

During commitment and differentiation, not all of the cells exposed to the morphogen showed ALP activity (Fig 3.4) nor did all of the cells lose the expression of myogenin (Fig 3.5, 3.7). This heterogeneity is in agreement with previous experiments that used BMP- 2 and 4 to induce osteogenesis ((Katagiri *et al.*, 1994; Katagiri *et al.*, 1997; Maeda *et al.*, 2004). The heterogeneity of cell populations is a well known fact. Isogenic cell populations may show different phenotypes. In general, variability may be due to microenvironment and/or

differential molecular distribution that cause variability in the regulation of gene expression (Portle *et al.*, 2007). More specifically, muscle satellite cells that include the C2C12 phenotype, have been described as heterogeneous and said to present functional disparities.

Upon serum limitation, about 50% of the C2C12 progenitors proceed to terminal differentiation marked by the expression of MyoD, myogenin and cellular fusion while cells whose level of MyoD remains low (MyoD negative cells) remain undifferentiated, creating a pool of reserve cells that retain their myogenic potential (Yoshida *et al.*, 1998). Also, the C2C12 line in particular contains a minor population of cells called side population (SP) that behave like true stem cells (Benchaouir *et al.*, 2004). Those cells may behave differently upon BMP-2 challenge. Although not well documented, the lack of homogeneity in response to BMP-2 may be attributed to the MyoD negative cells and/or the SP.

Among the factors affecting the BMP-2 response, interaction with the receptor(s) plays a key role and may cause differences in commitment within the cell population. BMP-2 binds type I receptors (BMPR-IA and -IB) with high affinity (Hassel *et al.*, 2004) and BMPR-II weakly unless co-expressed with BMPR-I (Gilboa *et al.*, 2000). The number of receptors per cell creates differences. Given the complexity of receptor complex formation, activation and function, many unknown interactions define the fine tuning of the signal initiated by BMP-2.

In this project, 50ng/ml of BMP-2 increased the level of ALP activity in a small fraction of the cell population but was not sufficient to repress the myoblastic lineage differentiation program (not shown) and to sustain osteoblastic differentiation in the whole cell population. I hypothesize that at this concentration BMP-2 binds with high affinity to preformed receptor complexes (if present, since the contribution of the Smads pathway to ALP activity increase was low) that through Smads increase the ALP activity level due to the

osteoblastic commitment of some cells. BMP-2 may also bind with high affinity BMPR-I that then recruits the BMPR-II to finally form BMP-2 induced receptor complexes that activates p38 also boosting the level of ALP activity. At this BMP-2 concentration the number of low affinity receptors bound by BMP-2 is probably too low to have an effect on the majority of the cells that therefore do not transdifferentiate. Under the same treatment (50ng/ml of BMP-2), the expression of myogenin monitored by immunofluorescence was very similar to the one observed in the absence of BMP-2. These results clearly show that this concentration of BMP-2 is insufficient to repress the expression of myogenin despite of the fact that ALP activity was higher than in control cells. Thus under our experimental conditions, when C2C12 cells were exposed to 50ng/ml BMP-2, the commitment switch was not activated.

Conversely at a dose of 300ng/ml (23nM), BMP-2 caused sufficient repression of the default myoblastic cell differentiation program (Fig 3.5, 3.7 BII) allowing for the transdifferentiation and sustained osteoblast phenotype. This dose is considered high when compared to the amounts used for other growth factors with C2C12 cells; for example, basic fibroblast growth factor (bFGF) at 1nM, insulin-like growth factor1 (IGF-1) at 3nM (Milasincic *et al.*, 1996) and TGF-β1 at 0.39nM; (Lee *et al.*, 2000). At the high dose, BMP-2 possibly binds the high affinity receptors plus a much higher number of low affinity BMPR-II, further recruiting type I receptors to form a receptor complex to finally assist effectively in the expression of the osteoblastic lineage. BMPR-IB would be the type I receptor best fitted in this scenario since it transmits signals for the osteoblastic lineage whereas BMPR-IA induced adipocytic differentiation in 2T3 mesenchymal precursors (Chen *et al.*, 1998).

A high and sustained level of BMP-2 is needed to achieve the highest levels of transdifferentiation of the majority of the C2C12 population. A high turnover rate for BMP-2

and/or BMP-2-receptor complexes has been reported. By qualitative observations and spectrofluorimetry Rauch *et al.*, (2002) observed significant cytoplasmic internalization of BMP-2 within 4-10 min at 37° C followed by fast degradation in the endosomal or lysosomal compartments. If degradation is the reason why high levels BMP-2 are needed, then it can be hyphothesized that commitment to transdifferentiation and myogenin repression acts as a switch (Vinals and Ventura, 2004) that needs a high dose of BMP-2 to be initiated but can be maintained with a lower concentration of the morphogen. An initial high concentration of BMP-2 may be sufficient to trigger the switch to transdifferentiation if there are sufficient numbers of low affinity receptors or if enough receptors bind the ligand and activate Smad proteins before BMP-2/receptor internalization and degradation. After the switch is completed, this requirement may change. This may be the case under our culture conditions since as BMP-2 concentration decreases due to degradation; a sufficient level may be left to sustain the needed signal. Since BMP-2 completely loses its activity at or around 70h under our culture conditions, re-supplementation was needed to pursue and possibly complete the maturation of the cells already committed to the osteoblastic lineage. Under the appropriate conditions, the cells would possibly be lead further to irreversible terminal differentiation.

One of the important conditions for progression of muscle-determined cells towards differentiation is the establishment of a "community" dependent on cell density and the establishment of cell-cell contacts that allows cellular interaction (Messina *et al.*, 2005). Myoblasts' differentiation into mature muscle cells is regulated by muscle-specific transcription factors such as myogenin, MyoD, Myf-5, MRF4/herculin/Myf-6. In C2C12 progenitors, the transcriptional activity of these factors in the nucleus may be suppressed by BMP-2 through modulation of their co-regulators (Katagiri *et al.*, 1997), induction of a degradation pathway and in the particular case of myogenin shortening of its half life (Vinals

and Ventura, 2004) from 60 to 30 min (Herpin *et al.*, 2004). As mentioned in the preceding section, only part of the population opted for myoblastic differentiation under 5% FBS and no exposure to BMP-2 as explained by the heterogeneity of the C2C12 population.

Myogenin was expressed in C2C12 cells at day 3 (Fig 3.5 and 3.7A). The similar levels of myogenin found at day 3 in untreated and treated cells may be due to the fact that the cells had just reached the confluency needed to allow cell-cell contact. Reaching confluency and having good cell-cell contact would result in a higher expression of the protein in untreated cells and the repression of transcription of the myoblastic genes in BMP-2 treated cells. The myoblastic gene repression in this project caused by BMP-2, affects not only myogenin transcription but also other muscle determination genes such as MyoD, herculin and myf-5 (Murray *et al.*, 1993). Myogenin, when detected by WB, showed as double bands. The upper band has been reported to be the hyperphosphorylated state of the protein and the lower band the non- phosphorylated state (Katagiri *et al.*, 1997).

It was unexpected to detect the presence of myogenin at day 6 in cells exposed to 300ng/ml of BMP-2. This indicates that although its transcription is mostly repressed, there is still transcription of the myogenin gene in some cells. This again may be attributed to C2C12 heterogeneity reported by Yoshida *et al.*, (1998).

Myogenin has been reported mainly in the nucleus but also in the cytoplasm of differentiated myotubules derived from C2 cells (Brennan and Olson, 1990). The sublocalization of myogenin in this project during the time of experimentation was mainly cytoplasmic, suggesting that the cells were not differentiated, although some BMP-2 untreated cells showed a nuclear localization of the protein, indicating a difference in the commitment/differentiation stage among the cell population.

The Kinetics of Transdifferentiation

In this project the processes of commitment and phenotype maintenance were studied through the addition and timely withdrawal of BMP-2, an area of research not previously studied in detail. Based upon measurements of increased levels of ALP during the first 72h of exposure to 300ng/ml of BMP-2 (Fig 3.8), most C2C12 progenitors have at least committed to the osteoblastic lineage between 24 and 48h. This is corroborated by the fact that the level of staining and back-extraction obtained at 72h is almost identical to that observed at 6 days. ALP mRNA has been reported to be present 48h after exposure of C2C12 progenitors to 300ng/ml of BMP-2. The level of ALP mRNA also increased with time (Katagiri *et al.*, 1994; Gallea *et al.*, 2001; Cheng *et al.*, 2003). With removal of BMP-2, some cells regressed to an ALP-activity free cell phenotype as seen by Katagiri *et al.*, (1994).

The cytoplasmic expression of myogenin found during the first 72h exposure to 300ng/ml of BMP-2 indicates that repression, if any, did not affect much the localization or expression level of the protein over this period of time (Fig 3.9 I). Myogenin remained at a cytoplasmic location at all time points and the strength of the signal did not vary much. This would indicate that repression of the myogenic lineage in the first 72h after exposure to BMP-2 is non-existent, very weak or is not manifested as tested. Myogenin localization and expression during "reversion" has not been previously reported under these conditions.

Exposure to BMP-2 for 6 hours followed by withdrawal and culture up to 6 days shows that reversion is achievable (Fig 3.9 II). The reversion is however influenced by the BMP-2 time exposure: cells treated with BMP-2 for 24h and then cultured under BMP-2 free conditions until day 6 showed lower myogenin expression than the cells exposed to the morphogen for 6h and also cultured under BMP-2 free conditions until day 6. Full reversion to the myoblastic lineage may be accomplished upon longer term cultures under myoblastic

conditions. When combining the results of myogenin expression and localization for increasing periods of time after BMP-2 exposure with those obtained after exposure and withdrawal of BMP-2, it can be concluded that exposure to BMP-2 does not immediately repress the expression of myogenin and that this repression takes place or is manifested at a time past 72h exposure to BMP-2. Also it can be said that the myogenin repression (if any) started by exposing the cells to BMP-2 for 6 hours is lost upon BMP-2 withdrawal and culturing to 6 days. In contrast, the myogenin repression started by exposing the cells to BMP-2 for 24 hours is still present after BMP-2 withdrawal and cell culturing to 6 days. It can be concluded that although the myogenin repression is not seen in the first 72 hours as per immunofluorescence, it started between 6 and 24 hours.

ALP activity and myogenin immunofluorescence data show that during the first 72h, cells exposed to BMP-2 do not show strong myogenin repression but exhibit very good ALP activity levels. This suggests that the repression of the myoblastic lineage and the enhancement of the osteoblastic one, although triggered by BMP-2 exposure, start at different times. Osteoblastic lineage enhancement is strong at 48h, while myogenin repression although seeming to start before 24 hours is not manifested (Fig 3.9I) in the short term. Upon BMP-2 withdrawal the behavior of the different populations composing the C2C12 line may be influenced by BMP-2 depletion. This potential change in behavior has not been explored.

The Behavior of the Smads during Commitment

Unphosphorylated Smads reside in the cytoplasm, BMP-2 causes the phosphorylation of R-Smads that once phosphorylated bind Smad-4 and then as a complex translocate to the

nucleus. Unphosphorylated Smads were found outside of the nucleus, in agreement with the expected results according to the reviewed literature. It was surprising however, and very interesting to find a very clear and strong signal of phosphorylated Smads at the pluripotent stage (absence of BMP-2 and 10% FBS) and under commitment (5% FBS) conditions in the presence and absence of BMP-2. The heterogeneity of the cell population did not seem to affect the signal strength or localization of those proteins. Confocal imaging and WB analysis proved that the pSmads seen at the nuclear level are indeed located inside the nucleus. Micrographic results also show the low level of unphosphorylated Smads (more clearly in the Smad-8 case) and help explain why the unphosphorylated Smads are beyond the sensitivity of WB detection.

The Smads-1, 5, 8 pathway can be induced by BMP-2 via ALKs 2, 3 or 6 and by TGF-β1 through ALK1 receptors. TGF-β1 is present in FBS and is also known to be secreted by C2C12 cells as an autocrine/paracrine growth factor. TGF-β1 may likely be phosphorylating one or more of the R-Smads belonging to the BMP pathway (Larsson and Karlsson, 2005). The consistent presence of pSmads in the nucleus may be further explained by the observed rate of nucleocytoplasmic shuttling discussed below. The potential effect of TGF-β1 on the BMP- Smads pathway might also explain the ALP activity in a small part of the C2C12 population in the absence of BMP-2 signal.

Smads shuttling between the nucleus and cytoplasm has been reported (Schmierer and Hill, 2005). In this project nucleocytoplasmatic shuttling of Smads was observed very seldom. This may indicate that Smads once phosphorylated quickly translocate to the nucleus where they accumulate, residing longer than in the cytoplasm, this may explain the extremely low signal of most unphosphorylated Smads when detected by WB. Schmierer and Hill, (2005) found that nucleocytoplasmic distribution of Smads, in both the absence and the

presence of a TGF-β-superfamily signal, is not static. Smads are continuously shuttling between the nucleus and the cytoplasm under both conditions. Accumulation of Smad-2 in the nucleus was triggered by the presence of TGF-β that caused exclusively the selective nuclear trapping of phosphorylated Smad-2. According to this information and the known autocrine secretion of TGF-β1 by C2C12 cells (Li *et al.*, 2004), TGF-β1 is a good candidate to be causing the phosphorylation and accumulation of a phosphorylated Smad in the nucleus of cells unexposed to BMP-2.

BMP-2 was found to be very effective in inducing the phosphorylation of some Smads. After 3h and at three days exposure, the morphogen raised the level of pSmads as detected with the pSmad-1, 5, 8 antibody by WB (Fig 3.17A). This confirms the presence of functional receptors on the cell surface and a functional pathway. The occurrence of protein bands in cells unexposed to BMP-2 indicates again the presence of an unknown factor that phosphorylates Smads, corroborating the results found with immunofluorescence.

Smads-4, 6 and 7 were expressed under the experimental conditions of this project. Little change was observed in their sublocalization.

The Relative Importance of the Smads Pathway during Commitment

In response to BMP-2 exposure, a transdifferentiation switch from myoblast to osteoblast phenotype is observed; however, other pathways may also contribute to further maturation. TGFβ-1 and p38 MAPK may contribute to ALP activation and influence myoblast differentiation depending on the cell stage and culture conditions (Katagiri *et al.*, 1994; Cabane *et al.*, 2003). Under our experimental conditions TGF-β1 and p38 MAPK were the most likely candidates contributing to transdifferentiation. A pharmacological approach was chosen to evaluate their potential involvement. SB431542 and SB202190 that

respectively inhibit the above mentioned pathways were used. Since these may alter proliferation, their effect on population size was tested prior to measuring the ALP activity. SB202190 was found to inhibit population increase over the 6 days of the experiment. This reduction was taken into account in the calculations of ALP activity. We did not however investigate whether all or a specific part of the cell population proliferation was targeted by the chemical. The contribution of the mentioned pathways in the commitment to transdifferentiation in cultures treated with 50 or 300ng/ml BMP-2 for 6 days was evaluated. The results from exposure to 50ng/ml of BMP-2 for 6 days suggested that both the TGF-β1 and P38 MAPK pathways act independently. However these differences were not significant. At 300ng/ml BMP-2 the 3 pathways contribute to the elevation of the ALP level. Their relative contribution was not similar or additive.

The Smads, p38 MAPK and TGF-β1 pathways through their potential elicitation via ALK1 and ALK5 receptors (the latter inhibiting the myoblastic phenotype) seem to collaborate to achieve the level of ALP activity found under the culture conditions. The fact that the contribution of the pathways is not additive tends to indicate that they merge or share communality at one point, a fact that has been shown for the Smads and p38 MAPK pathways that converge at the *Runx2* gene activation in mesenchymal precursos (Lee *et al.*, 2002). This has to be carefully evaluated since the cell may compensate for inhibition of one component of a pathway (Nishimaki-Mogami *et al.*, 2002; Campos *et al.*, 2004). Phosphorylation of the TGF-β1 receptor type I ALK5 is blocked by SB431542, thus any downstream reaction will be blocked, possibly including p38 MAPK which is also elicited independently from Smads by TGF-β1 (Ungefroren *et al.*, 2005). TGF-β1 is known to indirectly activate p38 MAPK via TAKI kinase (Laping *et al*, 2002). Therefore, blocking directly p38 MAPK phosphorylation via SB202190 would cause the same level of inhibition

assuming that Smad-2 and 3 also activated through ALK5 do not play any major role in the transdifferentiation process. For the first time, the greater than expected contribution of the TGF-β1 pathway and the lower than expected contribution of the Smads pathway to triggering ALP activity is quantified here. The two pathways also share commonality (Larsson and Karlsson, 2005). The fact that ALK1 may be activated by autocrine TGF-β1 or TGF-β1 in serum may explain the presence in the nucleus of phosphorylated Smads under proliferation conditions and under myoblastic commitment conditions. The C2C12 cell line is derived form a mouse presenting muscular dystrophy which may help explain the results. Myoblasts from patients with Duchenne muscular dystrophy release TGF-β1 as a growth factor to control proliferation and reduce differentiation and myotubule fusion (Melone *et al.*, 1999). It has been shown that C2C12 cells produce this autocrine factor (Li *et al.*, 2004).

It becomes evident that the interpretation of the results relates to the pathways cross talk. Thus, the complexity and dynamism of the TGF-β superfamily signaling (including BMPs) is further enhanced by the connection with other pathways (Larsson and Karlsson, 2005). Prominent MAPK pathways (Ras-MEK-ERK, MKK4-JNK, and MKK3-p38) may affect the Smads pathway in different ways, e.g., through repression of receptors' expression and/or increasing co-repressors levels (Massague, 2000).

Conclusions

1- Commitment and transdifferentiation of C2C12 myoblastic progenitors to the osteoblastic lineage can be achieved under this project's cell culture conditions.

2- A fraction of the cells do not commit to either the myoblastic or osteoblastic lineage, showing the heterogeneity of the cell population.

3- Intranuclear sublocalization of pSmads-1, 5 or 8 was observed almost at all times of the experimentation under different treatment conditions including the proliferative pluripotent stage. Since nuclear localization occurs only following phosphorylation of the Smad proteins, it means that the Smads proteins are phosphorylated in culture by an unidentified factor (likely TGF-β1) even in the absence of BMP-2.

4- pSmad-1 shuttling was observed during kinetics analysis upon BMP-2 challenge. Nuclear import quickly follows BMP-2 treatment while nuclear export may be slow resulting in the accumulation of the phosphorylated Smad species in the nuclei.

5- Transdifferentiation to the osteoblastic lineage starts as early as 6h when cells growing under low mitogenic conditions are exposed to 300ng/ml of BMP-2. The transdifferentiation process seems to be irreversible before or at 24h at least for the following 6 days in culture.

6- The ALP activity level resulting from the experimented BMP-2 doses may reflect the presence of operational high and low affinity receptors of BMP-2

7- A pharmacological approach led us to conclude that the BMP-2 elicited Smad pathway was not the main contributor to the increased ALP activity of maturating osteoblasts. In addition, the experiments unveiled that TGF-β1 and p38 MAPK share part of their pathways in the induction of ALP activity.

Future Work

This thesis raises several interesting questions. A question that remains unanswered involves the pSmads: Since the signal of pSmads is present and as expected located in the nucleus of C2C12 pluripotent progenitors and committing C2C12 unexposed to BMP-2, why is this signal not sufficient to repress the myoblastic phenotype and enhance the osteoblastic

one? Our semi quantitative results (WB) showing an increment in pSmads expression upon BMP-2 treatment. This points at a pSmads-1, 5, 8 expression threshold under which transdifferentation would not be completed. In addition, the issue of heterogeneity should be investigated. The observed heterogeneity suggests that osteoblastic transdifferentiation (as assessed via ALP activity) and myoblastic phenotype evolution are not complete over the duration of the experiments and may require more specific conditions to bring the cells to maturation and terminal differentiation. This is also reflected by residual myogenin expression at high dose BMP-2 treatment. Given the interesting and novel information found during this project, the following is proposed to complement the data:

1- To standardize a method to lower the concentration of BMP-2 needed to induce commitment to transdifferentiation. A preliminary experiment has been done in an effort to determine whether endocytosis as mentioned by Rauch *et al.*, (2002) was involved in the high amount of BMP-2 needed for transdifferentiation. Hypotonic shock (6:4 and 7:3 parts of 0% medium with BMP-2 and water for 15 and 30min and posterior culture in BMP-2 containing medium) was given to the C2C12 established cells to observe the impact on ALP activity levels after BMP-2 treatment. No significant improvement in ALP activity levels was seen with the treatments.

2- Properly optimize the back-extraction procedure.

3- To more accurately determine the time of activation of the commitment switch to transdifferentiation upon BMP-2 exposure and the sustainability of the acquired osteoblastic phenotype in order to manipulate and optimize the use of BMPs.

4- To explore in depth the effect of BMP-2 withdrawal in long term experiments in transdifferentiated C2C12 cells, to see if full reversion can be achieved. This would yield

data that can be used in planning *in vivo* experiments for potential therapeutic evaluation of adult progenitor xenografts.

5- To investigate more in depth the different phenotypes present in the C2C12 cell line and their fate before and after BMP-2 withdrawal. The information obtained can be used to choose specific cells from the C2C12 population that can be further characterized.

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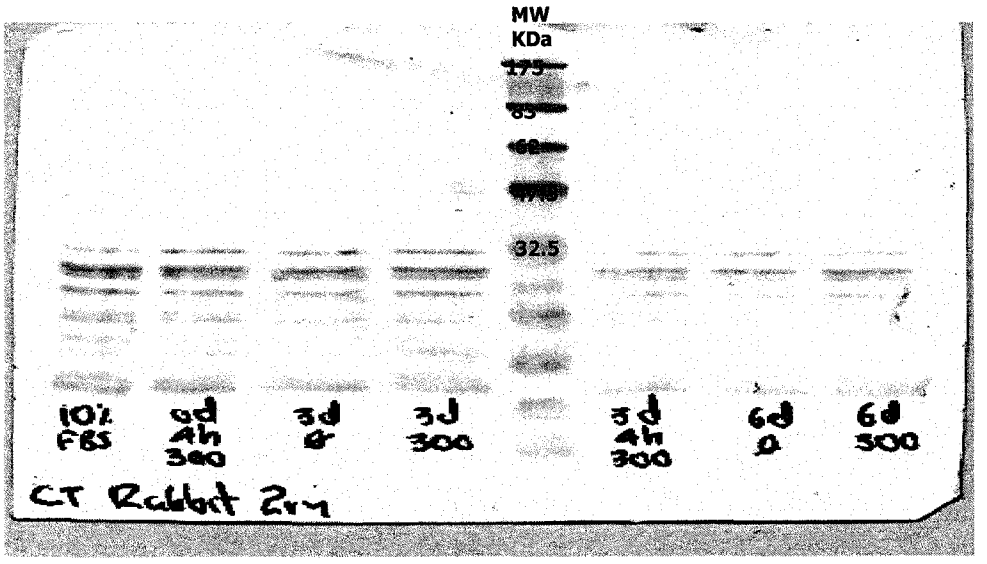
Appendix A

1.0 Complete Western Blots.

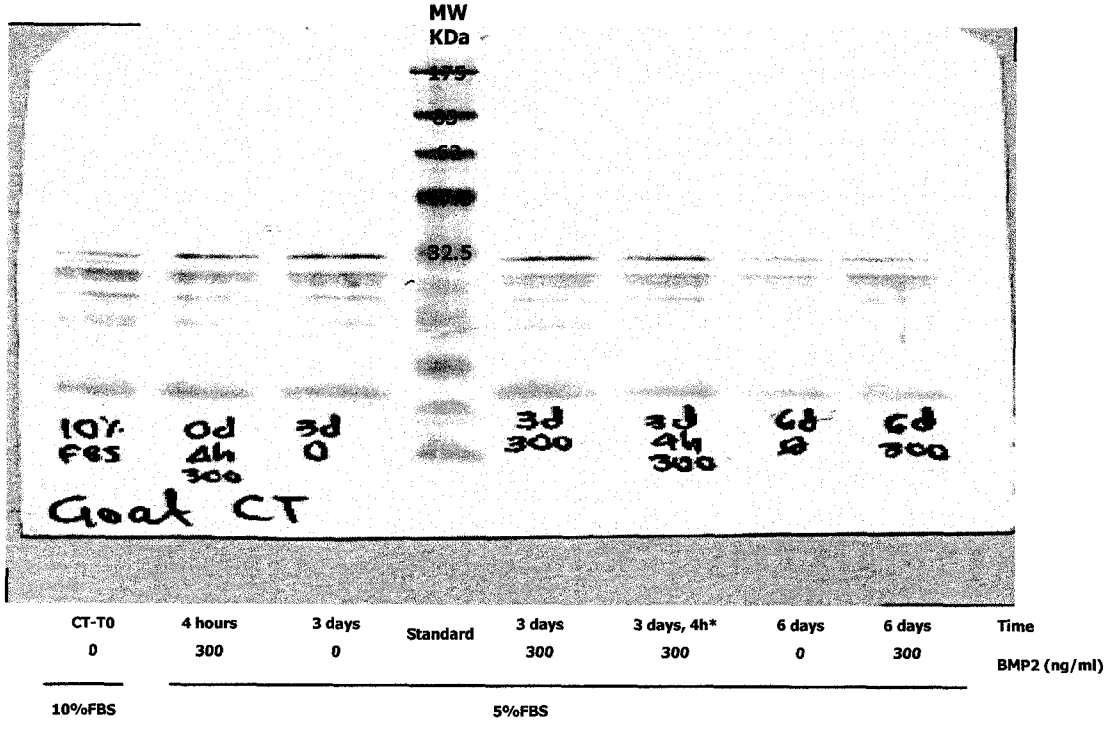
1.1 Goat Anti-rabbit Secondary Antibody Control



CT-T0	4 hours	3 days	3 days	Standard	3 days, 4h*	6 days	6 days	Time
0	300	0	300		300	0	300	BMP2 (ng/ml)
10%FBS				5%FBS				

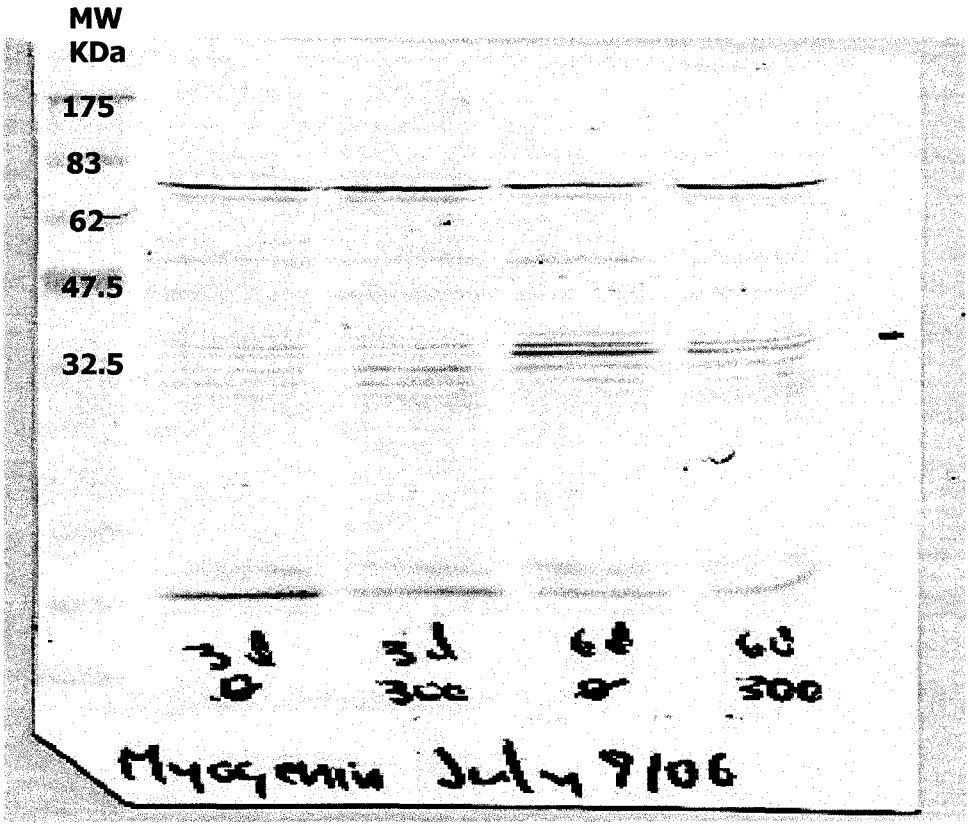
Goat anti-rabbit secondary antibody. C2C12 cells were cultured under the specified conditions. Extracts were prepared and 30ug of protein were loaded, separated by electrophoresis, transferred onto nitrocellulose membranes and detected with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Jackson). Duplicates were performed. CT-T0 cells were extracted after overnight settling in subculturing medium (10% FBS without BMP-2). The other treatments were started after cell settling by changing to commitment medium (5%-FBS with or without 300ng/ml of BMP-2) and incubated for the times indicated prior to processing. In the case of cells under 5% FBS treatment 3 days, 4h* with 300ng/ml of BMP-2, at 3 days the medium and BMP-2 were changed for fresh ones. Four hours later, the cells were extracted as per Materials and Methods.

1.2 Donkey Anti-goat Secondary Antibody



Donkey anti-goat secondary antibody. C2C12 cells were cultured under the specified conditions. Extracts were prepared and 30ug of protein were loaded, separated by electrophoresis, transferred onto nitrocellulose membranes and detected with donkey anti-goat alkaline phosphatase-conjugated secondary antibody (Jackson). Duplicates were performed. CT-T0 cells were extracted after overnight settling in subculturing medium (10% FBS without BMP-2). The other treatments were started after cell settling by changing to commitment medium (5%-FBS with or without 300ng/ml of BMP-2) and incubated for the times indicated prior to processing. In the case of cells under 5% FBS treatment 3 days, 4h* with 300ng/ml of BMP-2, at 3 days the medium and BMP-2 were changed for fresh ones. Four hours later, the cells were extracted as per Materials and Methods.

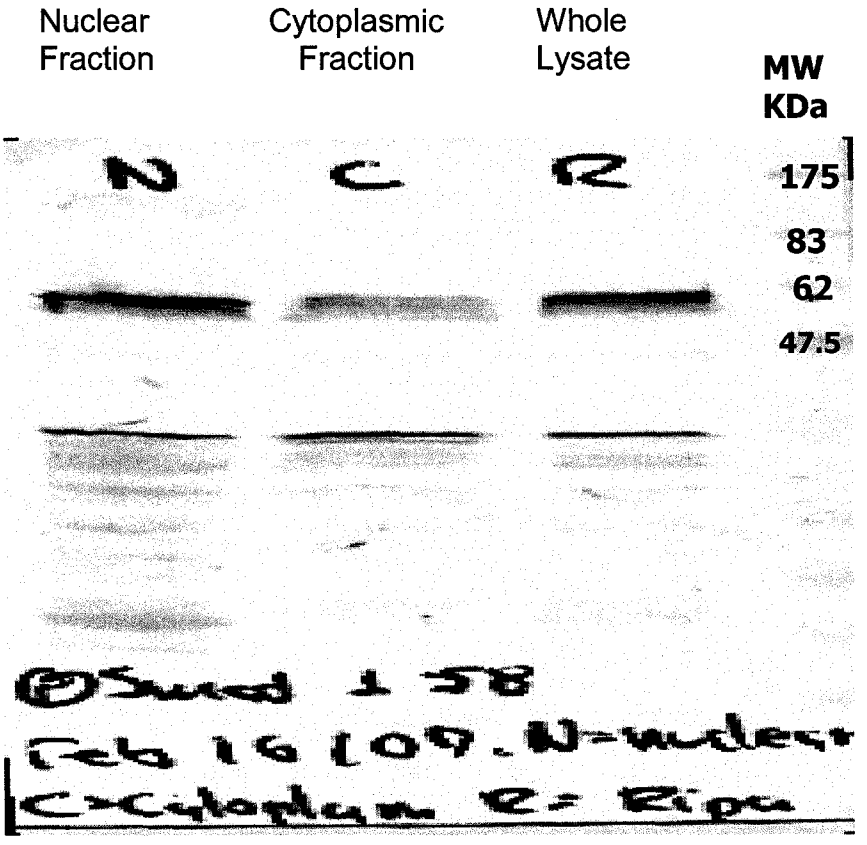
1.3 Changes in Myogenin Expression in Response to Commitment.



Standard	3 days	3 days	6 days	6 days	Time
	0	300	0	300	BMP2 (ng/ml)
	5%FBS				

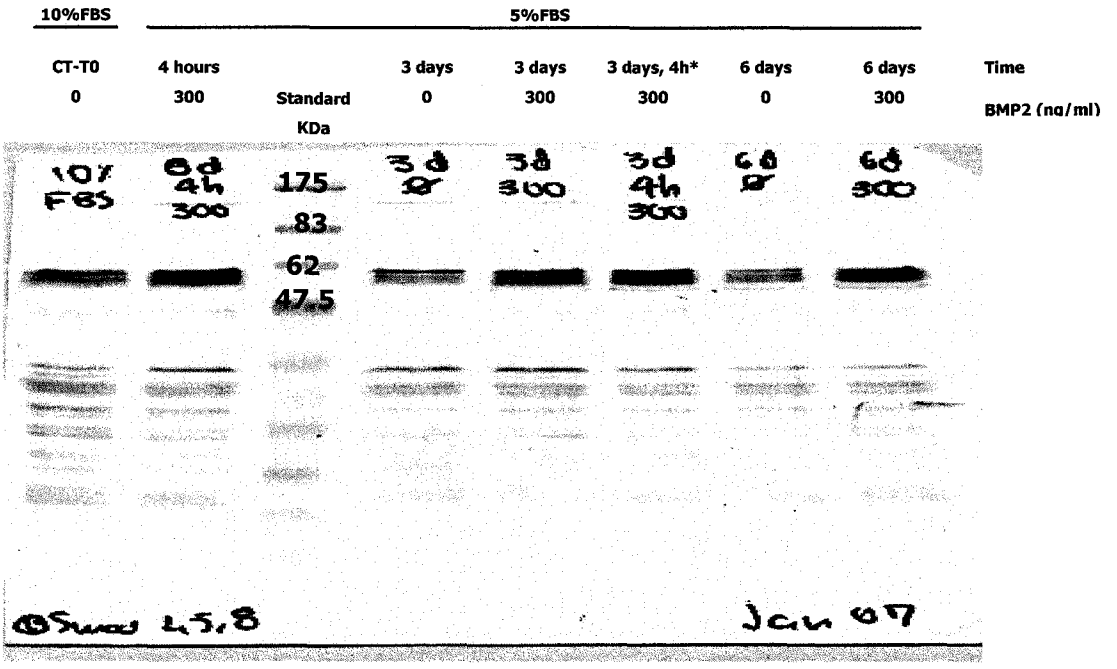
Changes in myogenin expression in response to commitment. C2C12 cells were cultured in 5% FBS supplemented with 0 or 300ng/ml of BMP-2 for 3 or 6 days. Cells lysates were prepared, and then 30ug of protein extract were electrophoresed through SDS/polyacryamide gels, transferred to membranes and probed with an anti-myogenin antibody as described in Materials and Methods section 2.5. For unspecific binding, cellular lysates from all treatments were processed with the secondary antibody alone. Duplicate experiments were performed.

1.4 Nuclear Extraction Profile of pSmads-1, 5, 8



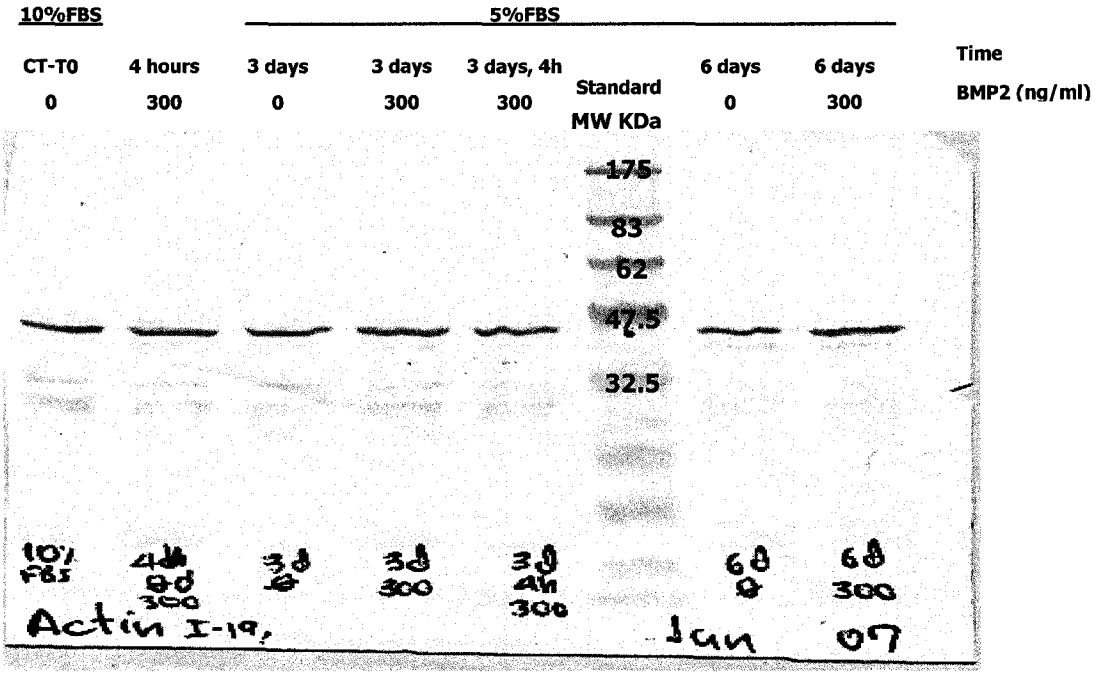
Nuclear extraction profile of pSmads-1, 5, 8. Cells were cultured for 3 days under osteogenic conditions. Extractions and WB of the enriched nuclear fraction, cytoplasmic fraction and whole cell extraction (RIPA) were performed as per Materials and Methods sections 2.4.2 and 2.5.4 with anti-pSmad-1, 5, 8 antibody 9511. Experiments were performed in duplicate.

1.5 pSmads-1, 5, 8 Expression under Different Culture Conditions.



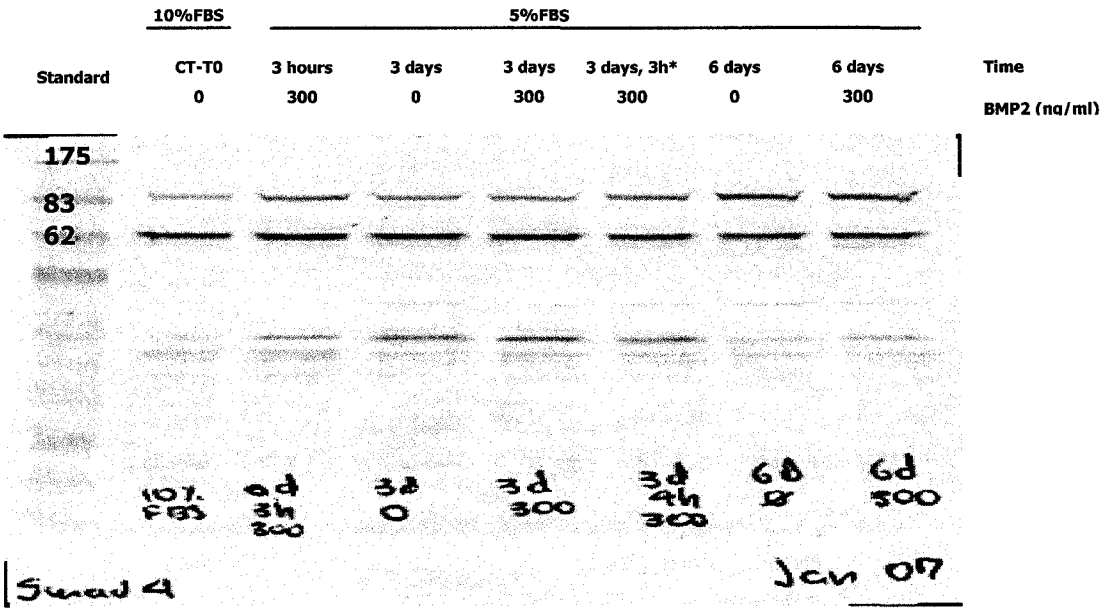
pSmads-1, 5, 8 expression under different culture conditions. C2C12 cells were cultured under the specified conditions. Extracts were prepared and 30ug of protein were loaded, separated by electrophoresis, transferred onto nitrocellulose membranes and detected with (A) the anti-pSmad-1,5,8 antibody 9511 (Cell Signaling) The standard lane in A shows a band at approximately 60KDa. Duplicates were performed. CT-T0 cells were extracted after overnight settling in subculturing medium (10% FBS without BMP-2). The other treatments were started after cell settling by changing to commitment medium (5%-FBS with or without 300ng/ml of BMP-2) and incubated for the times indicated prior to processing. In the case of cells under 5% FBS treatment 3 days, 4h* with 300ng/ml of BMP-2, at 3 days the medium and BMP-2 were changed for fresh ones. Four hours later, the cells were extracted as per Materials and Methods.

1.6 Actin as Loading Control



Actin expression under different culture conditions. C2C12 cells were cultured under the specified conditions. Extracts were prepared and 30ug of protein were loaded, separated by electrophoresis, transferred onto nitrocellulose membranes and detected with anti-actin antibody I-19 (Santa Cruz, actin is a house keeping gene used as loading control). The standard lane in A shows the 43KDa band. Duplicates were performed. CT-T0 cells were extracted after overnight settling in subculturing medium (10% FBS without BMP-2). The other treatments were started after cell settling by changing to commitment medium (5%-FBS with or without 300ng/ml of BMP-2) and incubated for the times indicated prior to processing. In the case of cells under 5% FBS treatment 3 days, 4h* with 300ng/ml of BMP-2, at 3 days the medium and BMP-2 were changed for fresh ones. Four hours later, the cells were extracted as per Materials and Methods.

1.7 Smad-4 Expression under Different Culture Conditions.

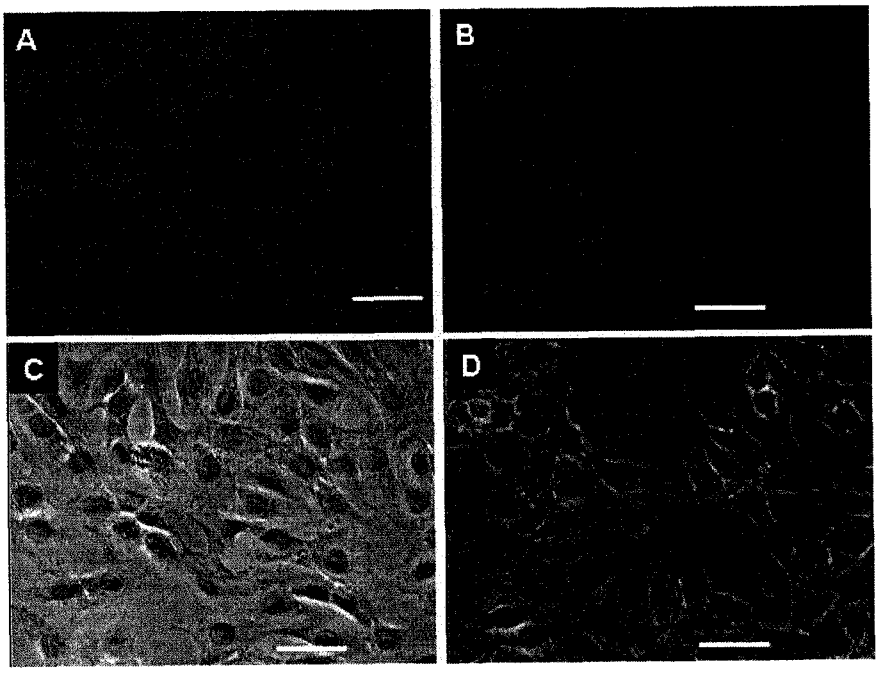


Smad-4 expression under different culture conditions. C2C12 cells were cultured under the specified conditions. Extracts were prepared and 30ug of protein were loaded, separated by electrophoresis, transferred onto nitrocellulose membranes and detected with (A) the anti-Smad-4 antibody (H-552, Santa Cruz). The blot shows a band at approximately 61KDa. Duplicates were performed. CT-T0 cells were extracted after overnight settling in subculturing medium (10% FBS without BMP-2). The other treatments were started after cell settling by changing to commitment medium (5%-FBS with or without 300ng/ml of BMP-2) and incubated for the times indicated prior to processing. In the case of cells under 5% FBS treatment 3 days, 4h* with 300ng/ml of BMP-2, at 3 days the medium and BMP-2 were changed for fresh ones. Four hours later, the cells were extracted as per Materials and Methods.

Appendix B

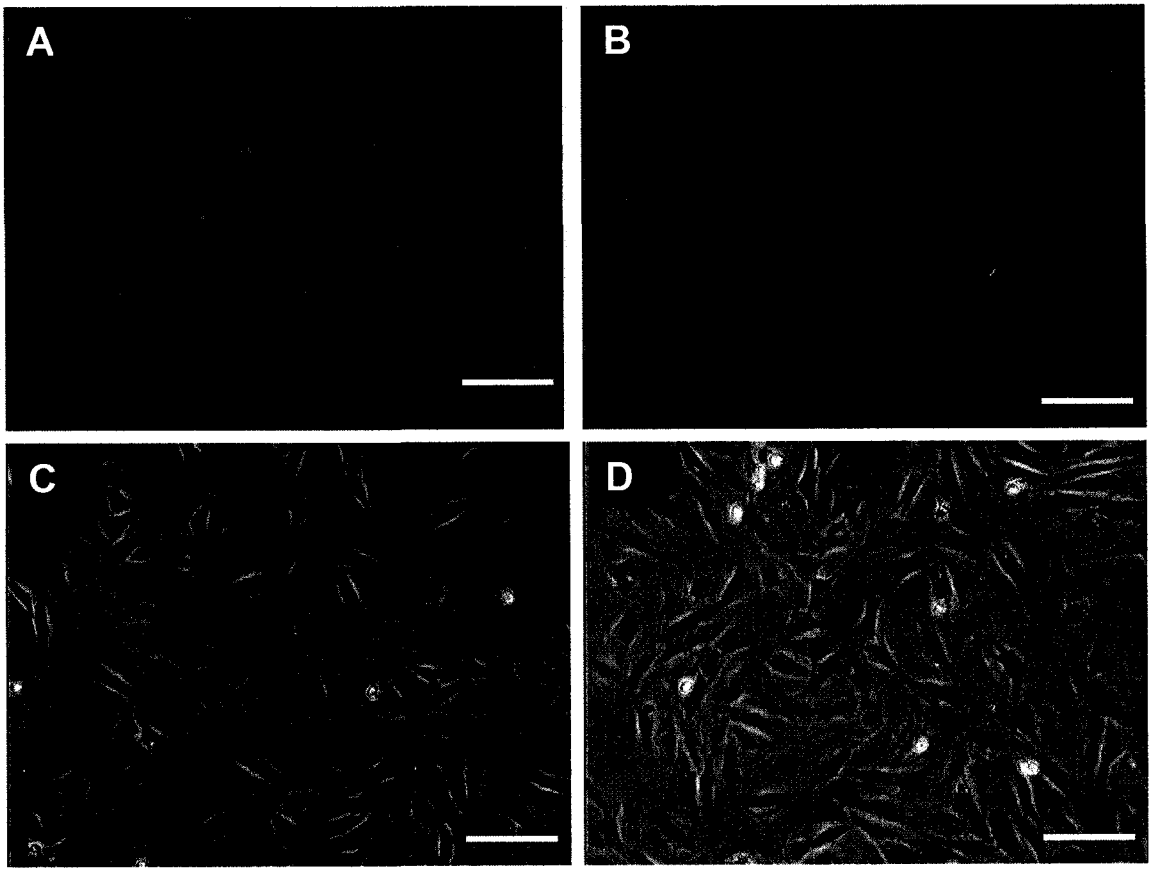
1.0 Immunofluorescence

1.1 Smads Shuttling



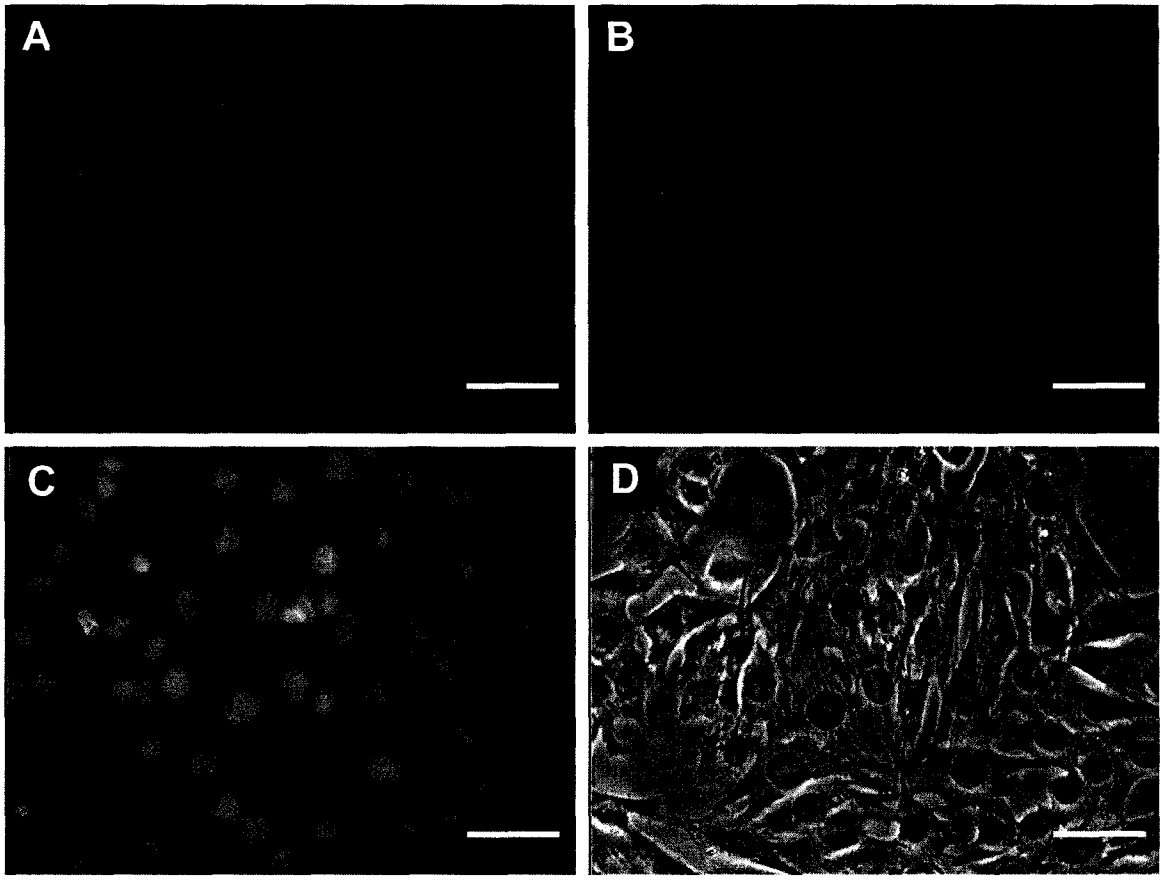
Shuttling of pSmad-1 under differentiation conditions. C2C12 were cultured under differentiating conditions (5% FBS, 300ng/ml BMP-2) for 30m (A, C) and 40m (B, D). Samples were prepared for microscopy as indicated in Materials and Methods section 2.6. The anti-pSmad-1 sc-12353 antibody (Santa Cruz) was used. Panel A shows the protein located possibly both in cytoplasm and nuclei with some perinuclear localization. Panel B possibly shows a protein more evenly located in both cytoplasm and nuclei hinting the shuttling of the protein cytoplasm ↔ nucleus. C and D are the phase contrast micrographs. The bars represent 25μm.

1.2 pSmad-1, 5, 8 Antibody Specificity



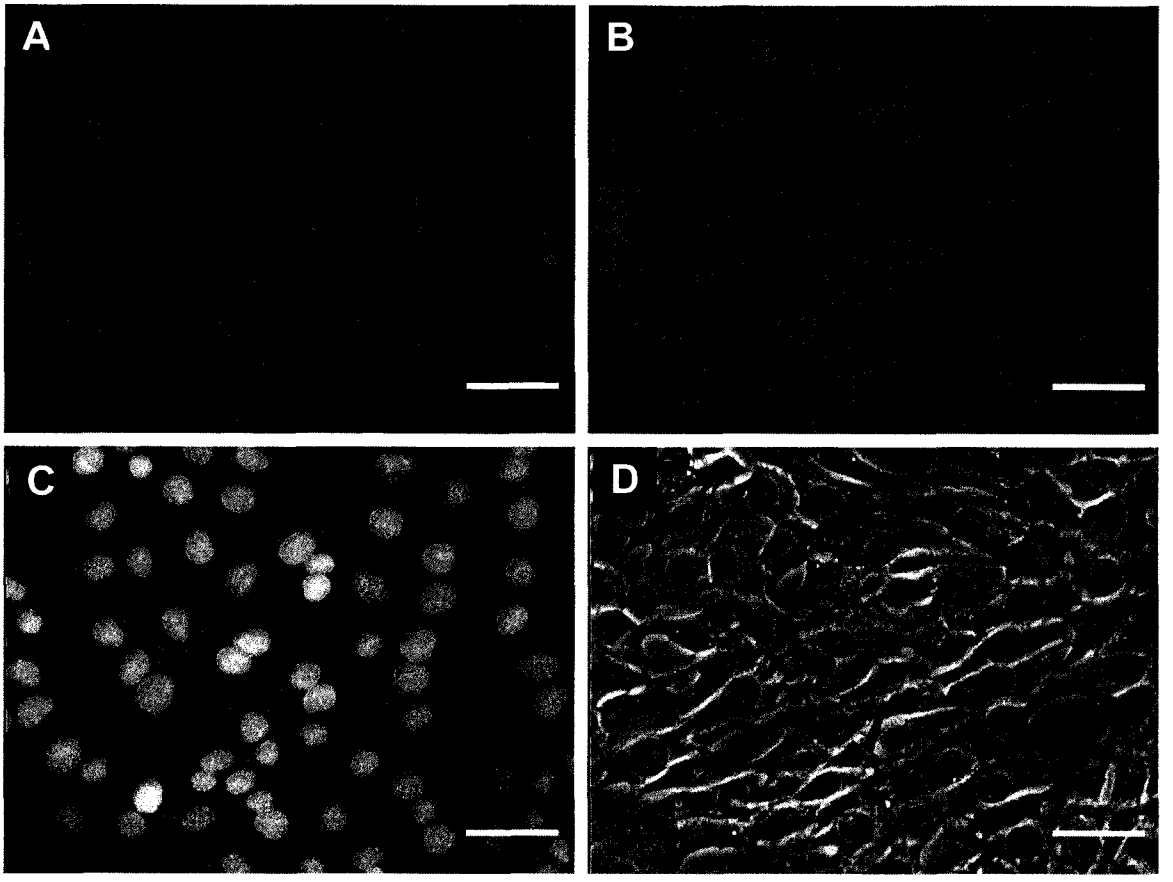
Specificity of the pSmad-1, 5, 8 antibody. C2C12 were cultured under starvation conditions (0.1% FBS) for 24h. Then cells were either prepared for microscopy (Panel A) as indicated in Materials and Methods section 2.6. Anti-pSmads-1, 5, 8 antibody 9511 (Cell Signalling) was used or exposed to 10% FBS for 15min (Panel B) and then prepared for microscopy as for panel A. Panels C and D correspond to the phase contrast micrographs. As can be seen, the exposure of the cells to FBS increased the signal from the antibody, indicating specificity. Experiment was done in duplicate. The bars represent 50μm.

1.3 Sublocalization of Smad-6



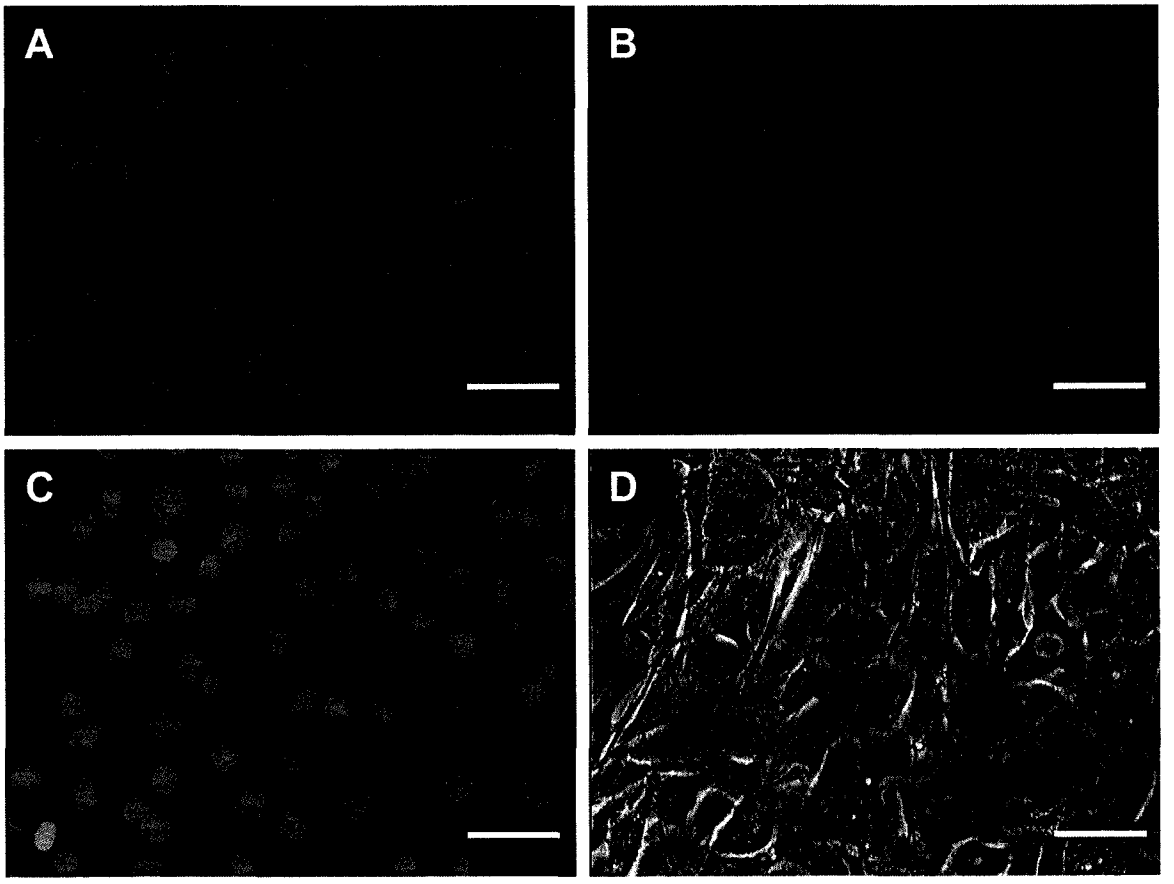
Cellular sublocalization of Smad-6 under commitment conditions. C2C12 were cultured under commitment conditions for 10min (in duplicate, in medium with 5% FBS) with or without BMP-2. Samples were prepared for microscopy as indicated in Materials and Methods section 2.6. The anti-Smad-6 antibody (H-150, Santa Cruz) was used. (A) DAPI nuclear staining; (B) immunolabeling of the protein(s); (C) Superposition of (A) and (B) to demonstrate possible co-localization (orange/yellow colour) at the nuclear level; (D) Phase contrast micrograph. Very similar results were obtained under all conditions (10% FBS, and 5% FBS with and without BMP-2) and time points. Duplicates were performed. Some pictures were enhanced to facilitate their interpretation; therefore, quantification of the protein is not feasible. The bars represent 25μm.

1.4 Sublocalization of Smad-7



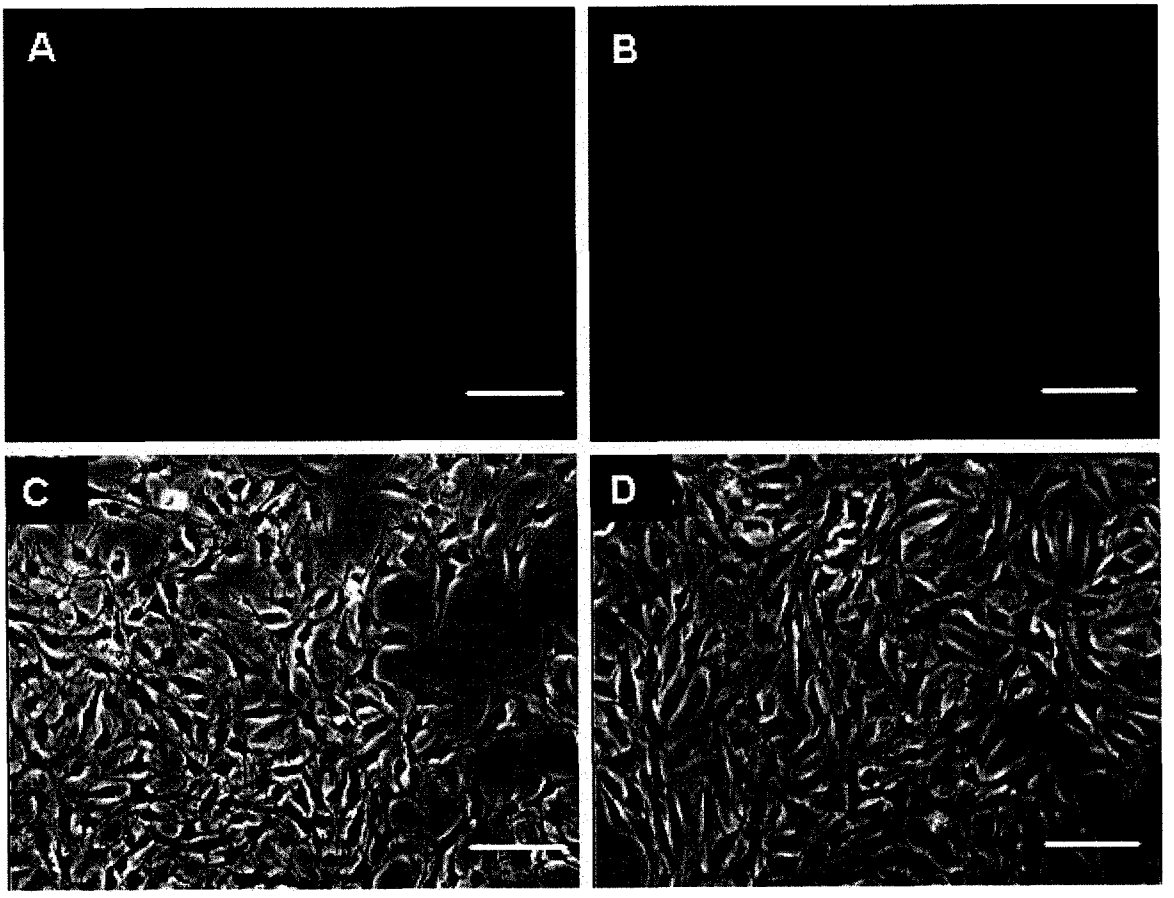
Cellular sublocalization of Smad-7 under commitment conditions. C2C12 were cultured under commitment conditions for 10min (in duplicate, in medium with 5% FBS) with or without BMP-2. Samples were prepared for microscopy as indicated in Materials and Methods section 2.6. The anti-Smad-7 antibody (H-79, Santa Cruz) was used. (A) DAPI nuclear staining; (B) immunolabeling of the protein(s); (C) Superposition of (A) and (B) to demonstrate possible co-localization (orange/yellow colour) at the nuclear level; (D) Phase contrast micrograph. Very similar results were obtained under all conditions (10% FBS, and 5% FBS with and without BMP-2) and time points. Duplicates were performed. Some pictures were enhanced to facilitate their interpretation; therefore, quantification of the protein is not feasible. The bars represent 25μm.

1.5 Sublocalization of Smad-4

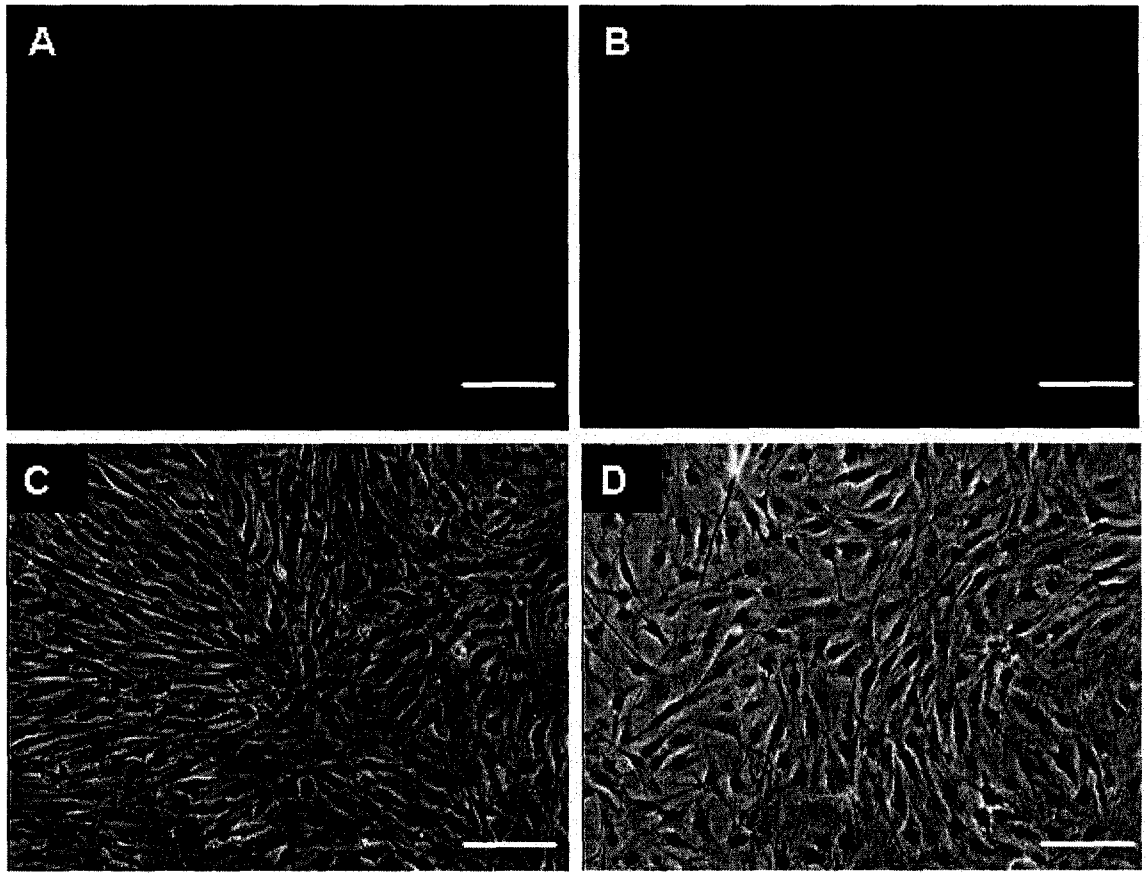


Cellular sublocalization of Smad-4 under commitment conditions. C2C12 were cultured under differentiating conditions for 3d (in duplicate, in medium with 5% FBS, no BMP-2). Samples were prepared for microscopy as indicated in Materials and Methods section 2.6. The anti-Smad-4 antibody (4 H-552, Santa Cruz) was used. (A) DAPI nuclear staining; (B) immunolabeling of the protein(s); (C) Superposition of (A) and (B) to demonstrate possible co-localization (orange/yellow colour) at the nuclear level; (D) Phase contrast micrograph. Very similar results were obtained under all conditions (10% FBS, and 5% FBS with and without BMP-2) and time points. Duplicates were performed. Some pictures were enhanced to facilitate their interpretation; therefore, quantification of the protein is not feasible. The bars represent 25μm.

1.6 Secondary Antibody Controls



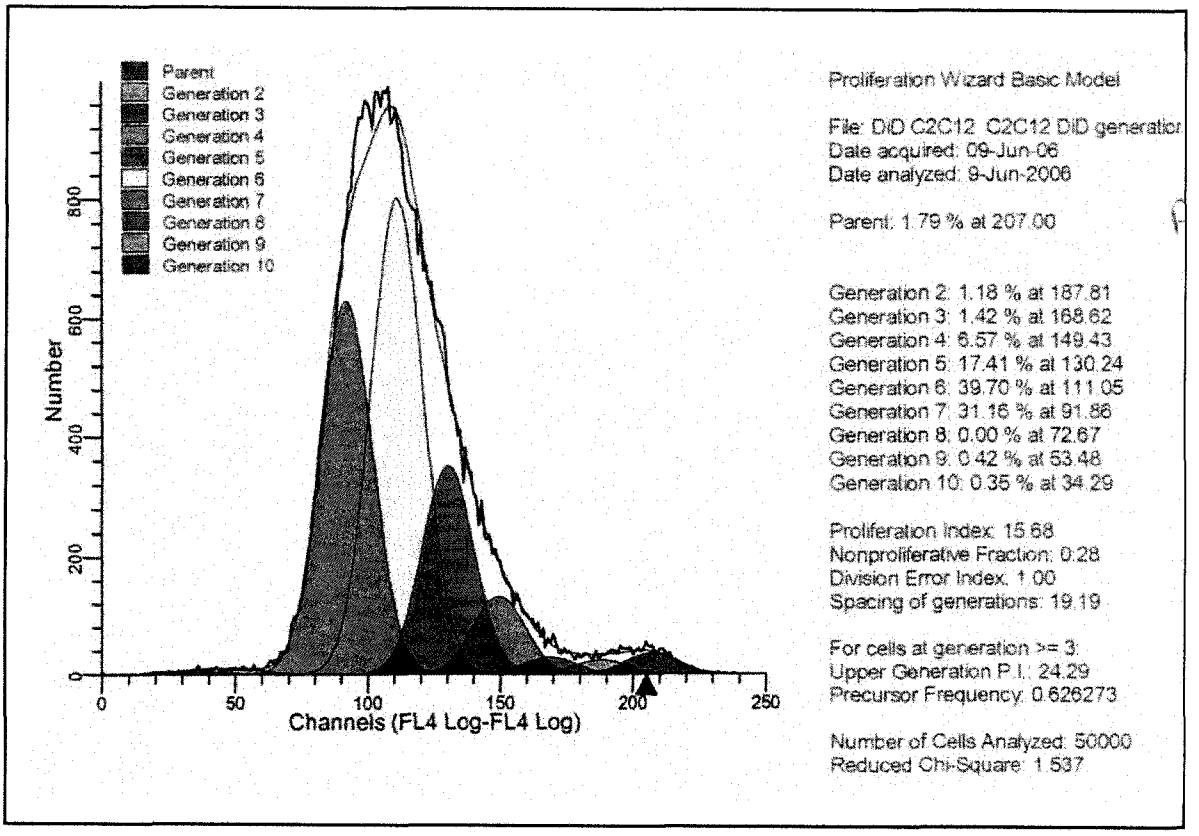
Immunofluorescence controls at day 3. C2C12 were cultured under differentiating conditions for 3d (in duplicate, in medium with 5% FBS, no BMP-2). Samples were prepared for microscopy as indicated in Materials and Methods section 2.6. Only secondary antibodies Alexa Fluor® 488 donkey anti-goat (panel A) and AlexaFluor® 488nm goat anti-rabbit (panel B) were used. C and D correspond to the phase contrast micrographs. Duplicates were performed. The bars represent 50μm.



Immunofluorescence controls at day 6. C2C12 were cultured under differentiating conditions for 6d (in duplicate, in medium with 5% FBS, no BMP-2). Samples were prepared for microscopy as indicated in Materials and Methods section 2.6. Only secondary antibodies Alexa Fluor® 488 donkey anti-goat (panel A) and AlexaFluor® 488nm goat anti-rabbit (panel B) were used. C and D correspond to the phase contrast micrographs. Duplicates were performed. The bars represent 50μm.

Appendix C

1.1 C2C12 Proliferation Rate and Index



C2C12 cell generations. C2C12 were cultured under passing conditions up to 96h (in duplicate, in medium with 10% FBS, no BMP-2). Samples were prepared for flow cytometry as per section 2.3.2 Proliferation Rate and Index. The daughter cell generations (up to generation 10) are depicted in the graph as generated by the ModFit (Verity Software House)