Regulation of Myogenesis by Cardiotrophin-1 and TGFβ signalling

Tetsuaki Miyake

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Abstract

Skeletal muscle differentiation is a process through which mono-nucleated myoblasts (MBs) form multi-nucleated myotubes (MTs). In this process, a highly orchestrated transcriptional regulatory network controls the program of muscle specific gene expression inducing specific genes in a spatially and temporally appropriate manner. Myogenic regulatory factors (MRFs) are muscle specific, sequence specific DNA binding transcription factors that play a central role in skeletal muscle differentiation. Two of the MRFs, MyoD and Myf5, are essential for myogenic lineage determination. MyoD is expressed in committed myogenic cells but the MBs do not differentiate until they receive appropriate cues. Induction of Myogenin (MyoG, another MRF), a critical downstream target of MyoD, is an absolute requirement for formation of myotubes. Therefore, myogenesis and particularly MyoD activity are inherently sensitive to external signals.

Here, we attempted to characterize the roles of two soluble factors in muscle differentiation, namely Cardiotrophin-1 (CT-1) and Tumour growth factor-β (TGFβ). CT-1 is a member of the IL-6 family of cytokines. Although CT-1 is highly expressed in skeletal muscle during embryo development and in the adult, the role of CT-1 in skeletal muscle had not been characterized. It was found that CT-1 inhibits muscle differentiation and regeneration through activation of MEK signalling. Activated MEK physically interacts with MyoD and interferes with MyoD's trans-activation properties. In the presence of CT-1, *myog* induction is inhibited without affecting MyoD and Myf5 protein levels suggesting that CT-1 maintains the undifferentiated state of MBs by activation of MEK (Chapter III).

TGFβ is the prototypic member of the TGFβ super-family of cytokines and a known potent inhibitor of myogenesis. We previously documented that an inhibitory Smad (I-Smad), Smad7, represses receptor regulated Smads (R-Smad) activation by TGFβ; yet, Smad7 can not reverse TGFβ's inhibitory effect on myogenesis. However, Smad7 enhances myogenesis by interacting with MyoD. These observations suggest that TGFβ inhibits muscle differentiation independent of R-Smad activation and that Smad7 promotes myogenesis independent of inhibition of R-Smad activity. Since Smad7 masks the TGFβ type I receptor from R-Smads in cytoplasm, we engineered a Smad7 fusion protein with a nuclear localization signal (NLS). This Smad7-NLS protein localizes predominantly in the nucleus and is incapable of inhibition of R-Smad activation by TGFβ signalling. However, the Smad7-NLS enhanced muscle differentiation to a similar degree as the wild-type Smad7. Smad7 interacted with the N-terminal region of MyoD in the nucleus. Since MEK also associates with the N-terminal of MyoD, we speculated that Smad7 enhances MyoD's trans-activation properties through attenuation of MEK's inhibitory effect. We demonstrated that Smad7 and MEK functionally antagonize each other in regulating MyoD's trans-activation properties. Therefore, Smad7 promotes myogenesis by enhancing MyoD's trans-activation properties in the nucleus independent of R-Smad inhibition in cytoplasm (Chapter IV).

Since Myostatin, another member of the TGFβ super-family, activates the ERK pathway, we hypothesized that TGFβ inhibits muscle differentiation through activation of MEK/ERK signalling instead of R-Smads. Blockade of R-Smad activation by a pharmacological inhibitor, SIS3, had no effect on the inhibitory effect

of TGFβ on myogenesis; however, a pharmacological MEK inhibitor, U0126, partially reversed the inhibitory effect of TGFβ. A known downstream target of MEK/ERK signalling c-Jun, which is an inhibitor of MyoD's trans-activation properties, was activated by phosphorylation and accumulated in the nucleus. As previously reported, ectopic expression of c-Jun strongly inhibited MyoD driven *myog* promoter reporter gene activity. Thus, TGFβ inhibits myogenesis by the MEK/ERK/c-Jun pathway independent of activation of R-Smads (Chapter V). These observations position MEK activation as a nexus for convergence of signals that regulate skeletal muscle differentiation; Conservation of this mechanism to regulate the key transcriptional machinery involved in myogenesis by a variety of cytokines indicates a pivotal role for this common cellular pathway in skeletal muscle ontogeny and physiology.

List of Abbrevations

AP-1	Activator protein-1
AMSH	Associated Molecule with the SH3 domain of STAM
AS-C	Achaete-Scute Complex
BA	Branchial Arche
BDNF	Brain-Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
bHLH	basic Helix-Loop-Helix
BMP	Bone Morphogenic Protein
BRCT	BRCA1 C'-Terminal
BRE	BMP Response Element
CBP	CREB Binding Protein
CD40	TNF receptor superfamily member-5
Cdc42	Cell division cycle 42
CDH15	M-Cadherin
CDK	Cyclin Dependent Kinase
CES	Core Enhancer Sequence
ChIP	Chromatin Immuno-Precipitation
CKI	CDK Inhibitor
CLC	Cardiotrophin-Like Cytokine
c-Met	hepatocyte growth factor receptor
CNTF	Ciliary NeuroTrophic Factor
COP9	Constitutive Photomorphogenic-9
CREB	Cyclic AMP Responsive Element Binding Protein
CRM1	exportin-1
CT	Calcitonin
CT-1	Cardiotrophin-1
CtBP	C'-terminal Binding Protein
CTGF/CCN2	Connective Tissue Growth Factor
Cxcl12	Chemokine (C-X-C motif) Ligand 12
DML	Dorsal Medial Lip
DN	Dominant Negative
DRR	Distal Regulatory Region
dsDNA	double stranded DNA
DSL	Delta, Serrate/ Jagged, and Lin-12 and Glp phenotype-2
Dusp1	Dual specificity phosphatase-1
E	embryonic day (mouse)
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMT	Epithelial-Mesenchymal Transition
ER81	Ets variant-1
ERK	Extracellular signal-Regulated Kinase
Evi1	Ectopic Viral integration site-1
FAK	Focal Adhesion Kinase
FERM	Four-point-one, Ezrin, Radixin, and Moesin
FGF	Fibroblast Growth Factor
FIK	Fibroblast Intermediate conductance calcium-activated potassium channel
FLRG	Follistatin-Related Gene
FoxO3	Forkhead box O3

FSTN	Follistatin
Furin	paired basic amino acid cleaving enzyme
GADD34	Growth Arrest and DNA Damege-34
GATA	GATA binding protein
GDF8	Growth Differentiation Factor-8
Gig1	Glucocorticoid-Induced Gene-1
gp130	Glycoprotein 130
GR	Glucocorticoid Receptor
Grb2	SH2 domain of Growth factor Receptor Bound protein
GSK3β	Glycogen Synthase Kinase-3β
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HER	v-erb-b2 erythroblastic leukemia viral oncogene homolog
Hes7	Hairy and enhancer of split-7
HGF	Hepatocyte Growth Factor
Hox	Homeobox
IBD	Inflammatory Bowel Disease
IFN	Interferon
IGF1	Insulin-like Growth Factor-1
IGFBP5	Insulin-like Growth Factor-Binding Protein-5
IL	Interleukin
Itch	Itchy E3 ubiquitin protein ligase homolog
Jab1/CSN5	COP9 constitutive photomorphogenic homolog subunit-5
JAK	Janus Kinase
JNK	c-Jun N'-terminal Kinase
kb	kilo-base
KLF10/TIEG1	Kruppel-Like Factor-10
LEF	Lymphoid Enhancer-binding Factor
LIF	Leukemia Inhibitory Factor
LIFRβ	LIF β-Receptor
LIM	α-actinin-2-associated LIM protein
LIMK1	LIM Kinase-1
LRP1	Lipoprotein-receptor Related Protein
MAML1	Mastermind-Like-1
MAPC	Multipotent Adult Progenitor Cell
MAPK	Mitogen-Activated Protein Kinase
MB	Myoblast
MCM1	yeast mating type decisions
MDSC	Muscle-Derived Stem Cell
MEF2	Myocyte Enhancer Factor-2
Mefgf10	Multiple epidermal growth factor repeat transmembrane protein
MEKK	MAPK Kinase Kinase
MH2	MAD (Mothers against decapentaplegic) Homology-2
MIP1β	Macrophage Inflammatory Protein-1β
MKKK7/MAP3K7	MAPK Kinase Kinase 7
MKP1	Mitogen activated protein Kinase Phosphatase-1
MMP	Matrix Metalloproteinase
MRF	Myogenic Regulatory Factor
MSTN	Myostatin
MT	Myotube

mTOR	mammalian Target Of Rapamycin
muSP	muscle-resident Side Population
Myf5	Myogenic Factor-5
MyoD	Myogenic Differentiation 1
MyoG	Myogenin
N-CoR	Nuclear receptor Co-Repressor
NES	Nuclear Export Signal
Neu	Neuro/glioblastoma derived oncogene homolog
NF-κB	Nuclear Factor-κB
NICD	Notch Intracellular Domain
NO	Nitric Oxide
OAZ	Zinc finger protein-423
OSM	*Oncostatin M*
P/CAF	P300/CBP-Associated Factor
$p21^{Cip-1}$	cyclin-dependent kinase inhibitor-1A
$p27^{Kip-1}$	cyclin-dependent kinase inhibitor-1B
p38MAPK	p38 Mitogen Activated Protein Kinase
$p57^{Kip-2}$	cyclin-dependent kinase inhibitor 1C
Pai1	Plasminogen activator inhibitor-1
Pax3	Paired box gene-3
Pax3-/-	Pax3 null
PBE	Proximal BMP-responsive Element
PDK1	Pyruvate Dehygrogenase Kinase-1
PH	Pleckstrin Homology
PI3K	Phosphatidylinostol 3-Kinase
PIAS	Protein Inhibitor of Activated STAT
Pin1	Peptidylprolyl cis-trans isomerase
PKA	Protein Kinase A
PKB/Akt	Protein Kinase B
PKC	*Protein Kinase C*
PP2A	Serine/threonine Protein Phosphatase 2A
PSM	Presomitic Mesoderm
Rac	Ras-related C3 botulinum toxin substrate (Rho family, small GTP binding protein)
Rb	Retinoblastoma protein
ROCK	Rho/Receptor for activated protein kinase C
R-Smad	Receptor Smad
Runx2	Runt-related transcription factor-2
SAD	Smad4 Activation Domain
SBE	Smad Binding Element
SCP	Small C'-terminal domain Phosphatase
SDF1	Stromal-Derived Factor 1
SH2	Src (v-src sarcoma viral oncogene homolog) Homology domain-2
Shc	*SH2 and collagen homology domain containin protein*
Shh	Sonic hedgehog
SHP2	SH2 domain containing tyrosine Phosphatase
SIK	Salt-Inducible Kinase
SIRT1	Sirtuin (silent mating type information regulation-2) homolog-1
SKIP	Ski Interacting Protein
Smad	Mothers against decapentaplegic homolog
Smurf	Smad ubiquitin Regulatory Factor

Sp1	trans-acting transcription factor 1
SRF	Serum Response Factor
STAM	Signal Transducing Adaptor Molecule (SH3 domain and ITAM motif)
STAT	Signal Transducer and Activator of Transcription
STRAP	Serine/Threonine kinase Receptor Associated Protein
SUMO-1	Small Ubiquitin-like Modifier-1
Swift	Smad wing for transcriptional activation
TAB	TAK1 Associated Protein
Tagln/Sm22α	Transgelin/Smooth muscle 22 protein
TAK1	TGFβ Activated Kinase-1
TAZ	Transcriptional co-activator with PDZ-binding motif
Tbx6	T Box family member-6
Tbx6	T Box family-6 protein
TCF	T-Cell specific Factor
TFE3	Transcription Factor binding to IGHM Enhancer-3
TGFβ	Tumour Growth Factor β
TGIF	TGFβ-Induced Factor homeobox
TNFSF12	Tumour Necrosis Factor (ligand) Superfamily member-12
TNFα	Tumour Necrosis Factor α
Tob1	Transducer of ErbB-2.1
TSS	Transcriptional Start Site
TWEAK	Tumour necrosis factor-like WEAK inducer of apoptosis
TYK2	Tyrosine Kinase-2
UV	Ultraviolet
VCAM1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VLA4	Integrin β1
VLL	Ventral Lateral Lip
WWTR1	WW domain containing Transcriptio Regulator-1
YB1	Y-Box protein-1

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I could not keep a promise. I am sorry about this, Joseph.

Finally, to my wife, kids, and parents, I ignored large portion of my responsibility, which you have picked-up. I will be more responsible..... I will try....

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Tetsuaki Miyake

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Chapter I; Literature Review

Chapter I; Literature Review

Anatomy of skeletal muscle development (myogenesis)

1.1. During embryo development; Somitogenesis to muscle differentiation

In vertebrates, skeletal muscle is an essential tissue to provide locomotion and proper posture for the organism. During embryogenesis, all skeletal muscle with the exception of some craniofacial and oesophageal muscles is generated from progenitor cells originating from somites (originally described as primitive segments). Somites are transient embryonic structures of segmented blocks of paraxial mesoderm which eventually differentiate and give rise to skeletal muscle, vertebrae, cartilage, tendon, and dermis. Somitogenesis starts at the anterior end of unsegmented presomitic mesoderm at embryonic day 8 (E8 in mouse) and proceed towards the posterior end at a constant interval in a symmetric manner on both sides of the neural tube (figure 1A). By E12-13, approximately 30 pairs of somite are formed during mouse embryo development (Buckingham et al., 2003; Ordahl and Le Douarin, 1992; Parker et al., 2003; Tajbakhsh and Sporle, 1998). This segmentation process is regulated by the segmentation clock, which is controlled by a molecular oscillation of the members of the Notch and Wnt signalling pathways and fibroblast growth factor (FGF) in the presomitic mesoderm (PSM) (Dubrulle et al., 2001; Jiang et al., 2000). This periodic formation of segmented somites from the PSM is regulated by the basic helix-loop-helix (bHLH) transcription factor Hairy and enhancer of split-7 (Hes7), which is essential for somitogenesis of the mouse embryo. The FGF signalling is required for *hes7* gene expression, and Hes7

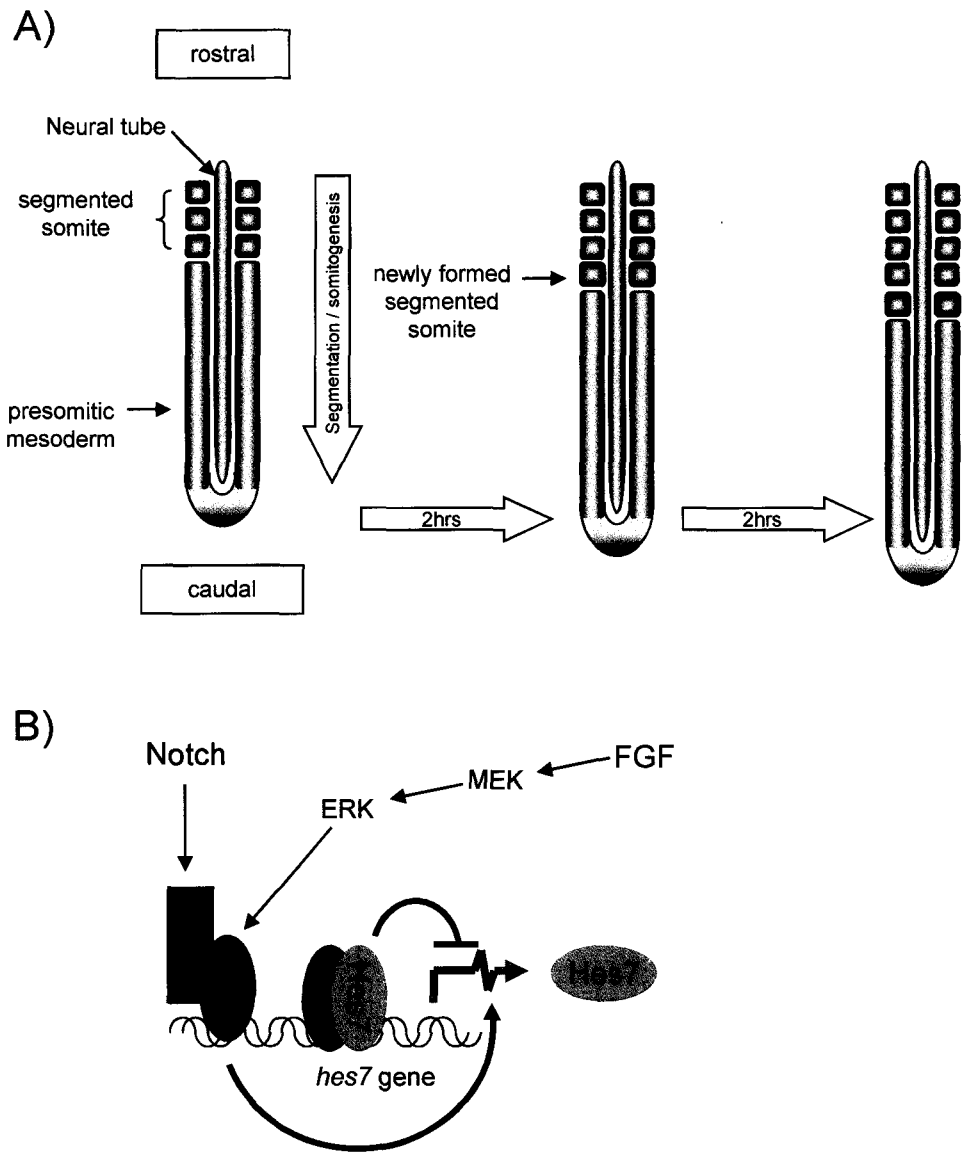


Figure 1. **Somitogenesis during mouse development** A) Somite formation B) Rbpj;recombination signal binding protein for immunoglobulin κ J region

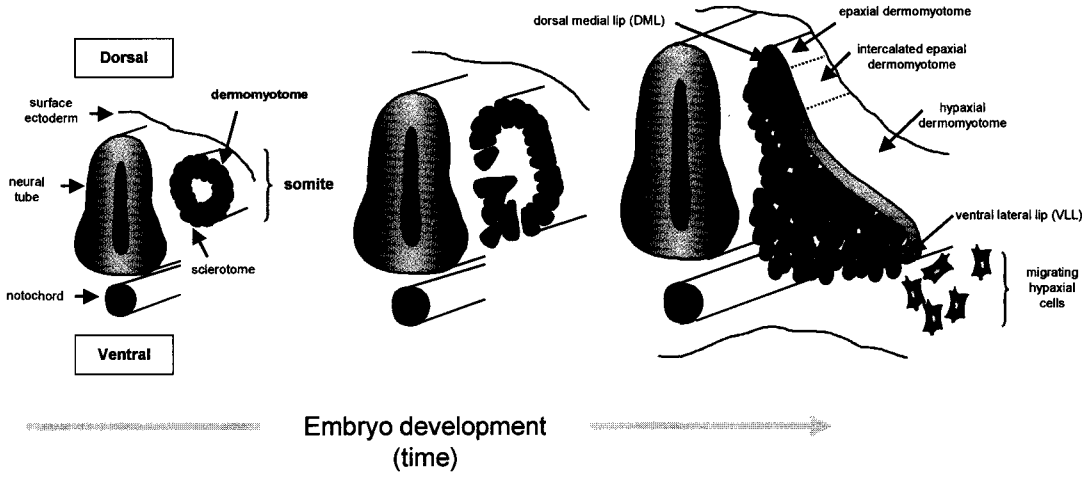
negatively autoregulates its own gene expression. The Notch signalling also activates *hes7* expression (figure 1B). Therefore, co-operation of FGF with the Notch signalling pathways generates an oscillation of *Hes7* expression resulting in the formation of a pair of segmented somites approximately every 2hrs during mouse embryo development (Feller et al., 2008; Hirata et al., 2004; Kageyama et al., 2009).

A newly formed segmented somite is a sphere of epithelial cells, which later gives rise to distinguishable groups of cells such as dermomyotome, myotome, syndetome, and sclerotome (figure 2A) (Brent et al., 2003; Brent and Tabin, 2002). The sclerotomes give rise to the cartilage and bones, and the syndetome develops into the tendons. The dermomyotome gives rise to the dermis and skeletal muscles (Marcelle et al., 1997; Ordahl and Le Douarin, 1992) (figure 2B). In detail, the deep back muscle originates from the epaxial myotome, the lateral trunk muscles are developed from the ventral lateral lip (VLL). Some of the VLL derived cells undergo epithelial-mesenchymal transition (EMT) and migrate to lateral regions contribute to the formation of the limbs, ventral wall, diaphragm and tongue muscles (Parker et al., 2003).

1.2. Skeletal muscle growth and regeneration in adults

Adult skeletal muscle can be regenerated in response to injury throughout life. To allow this remarkable ability, adult skeletal muscle stem cells have capabilities of 'self-renewal' to maintain the stem cell population and also 'differentiation' to regenerate multinucleated myotube (MT). There are mononucleated cells located

A)



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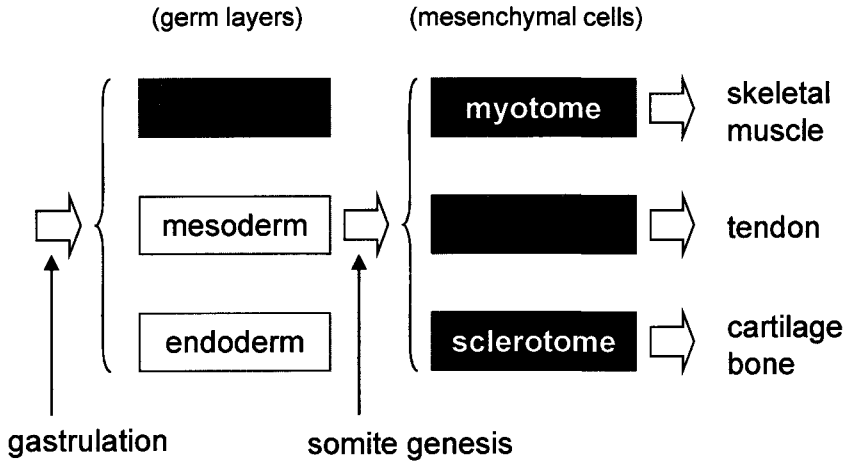

Figure 2. **Muscle formation in embryo development** A) mesenchymal cell lineage specification. Myotome (blue), syndetome (green), and sclerotome (purple). B) cell fate determination.

inside of the basal lamina but outside of the plasma membrane of the mature myofibers named 'satellite cells' which fulfill these roles (Mauro, 1961) (figure 11). Isolated 'satellite cells' were found to proliferate to expand their population and differentiate to form multinucleated myotubes *in vitro* (Bischoff, 1986). Although it has not yet been demonstrated definitively that all satellite cells are a skeletal muscle stem cells *in vivo*, at least a portion of the satellite cells in adult skeletal muscle has the progenitors of asymmetric self renewal and differentiation (Kuang et al., 2008).

The muscle satellite cells originally arise from the central dermomyotome during embryo development after generation of the primary myotome (figure 4). In a neonatal mouse, about 32% of nuclei associated with myofibers represent growth arrested satellite cells. However, in a mature adult mouse, approximately 6% of the nuclei belong to the satellite cells (Cardasis and Cooper, 1975). Therefore, it is not clear that these satellite cells in adults are of identical origin as the satellite cells in a newborn. In addition, recent analysis indicates adult skeletal muscle resident stem cells that are not satellite cells. For example, muscle-resident side population (muSP) (Asakura et al., 2002), muscle-derived stem cells (MDSCs), and multipotent adult progenitor cells (MAPCs) (Jiang et al., 2002) have been identified. The roles of these cells in muscle growth and repair need to be characterized. However, satellite cells are sufficient for complete musclefiber regeneration after extensive muscle injury *in vivo*; therefore, the satellite cells seem to contribute the great majority of the stem cell pool for regeneration or growth of adult skeletal muscle. Upon appropriate stimuli such as muscle injury or exercise,

the quiescent satellite cells re-enter the cell cycle, and these proliferating myoblasts divide asymmetrically and contribute to both repopulate quiescence satellite cell and fusing into and providing new myonuclei for myotubes (figure 11). Therefore, skeletal muscle regeneration and growth can be achieved without expense of the satellite cell population (Kuang et al., 2008).

1.3. Myoblast (MB) formation

Vertebrate skeletal myogenesis consists of three phases, embryonic, fetal, and adult myogenesis. Embryonic myogenesis (E10.5-12.5) determines the principal muscle pattern. Fetal myogenesis (E14.5-17.5) is important for muscle maturation and growth. Adult myogenesis is necessary for postnatal growth and damage repair (Biressi et al., 2007a). Accumulating evidence indicates that not only adult muscle progenitor cells but also prenatal muscle progenitor cells are not a homogeneous population (Asakura et al., 2002; Cao et al., 2003; Jiang et al., 2002; Olguin and Olwin, 2004). During each phase of myogenesis, a distinct population of myogenic precursor cells (myoblasts; MB) forms muscle. Therefore, they are characterized as embryonic MB, fetal MB, and satellite cell (figure 3). As mentioned above, the origin of the vast majority of skeletal muscle is the somitic dermomyotome. Prior to forming the dermomyotome, myogenesis starts in a newly formed somite, and the first terminally differentiated myocytes in the embryo generate from the dermomyotome later; the myotomal cells at both dorsal medial lip (DML) and VLL expand under the dermomyotome, withdraw from the cell cycle, elongate, and differentiate to form the myotome. Proliferating MBs arise from the central region

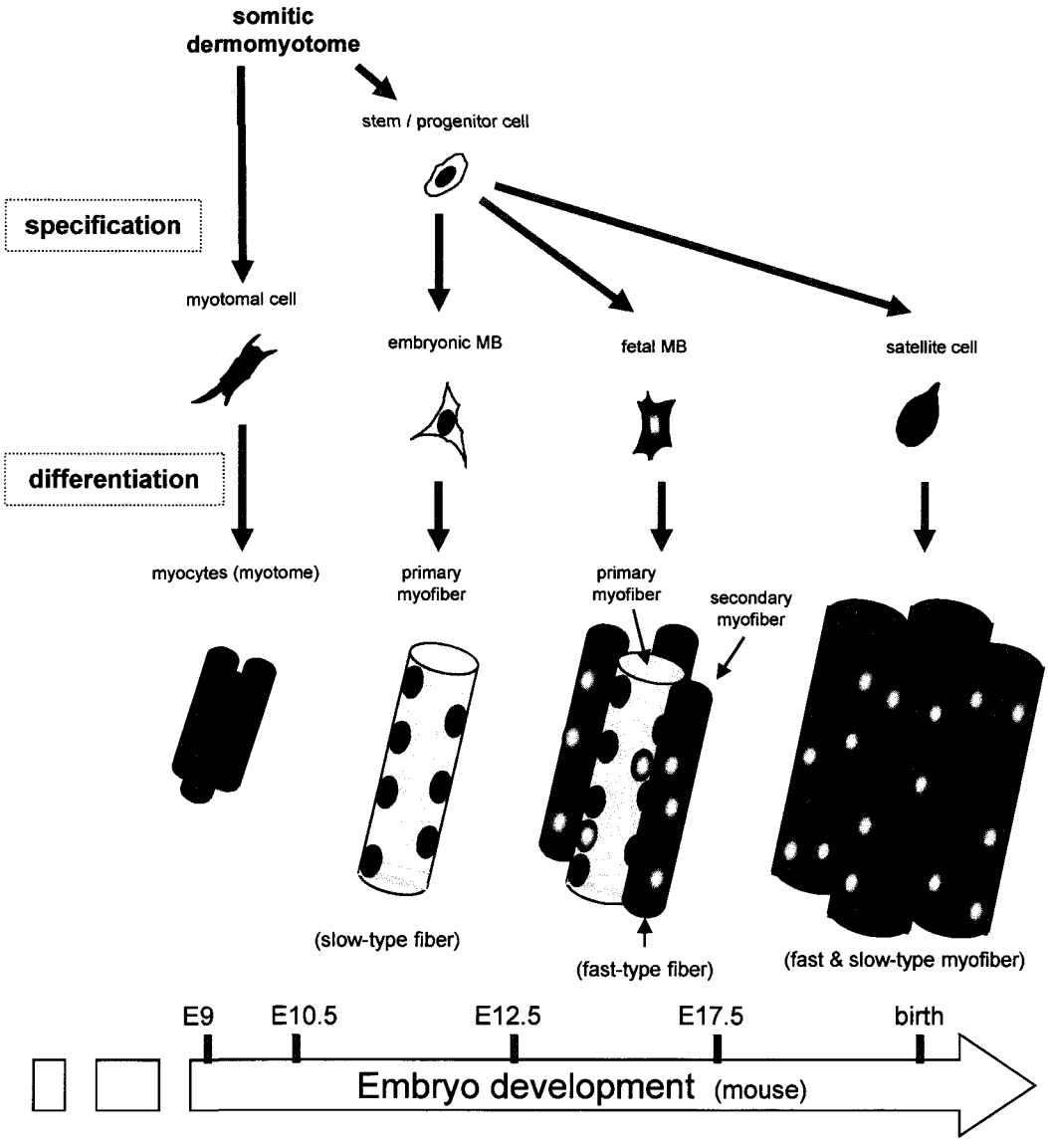

Figure 3. **Specification and differentiation of myogenic cells**

of the dermomyotome (figure 4). At approximately E11, it seems that embryonic MBs fuse to the myotome and form MTs (figure 3). At E14.5 to E17.5, secondary myogenesis takes place, in which fetal MBs fuse with each other and form secondary fibers and also a small portion of fetal MBs fuse to the primary fibers. At the end of secondary myogenesis (E16.5), slow-proliferating satellite cells can be identified between the basal lamina and the plasma membrane of the myofibers (Biressi et al., 2007a; Parker et al., 2003) (figure 11). The supply of new myonuclei is almost entirely relying on the progeny of the satellite cell for peri- and post-natal muscle growth. For the repair of damaged adult muscle, quiescent satellite cells are activated by appropriate stimuli and re-enter the cell cycle to produce progeny, which may fuse to each other to form new myotubes or fuse to pre-existing myofibers. There are three types of MBs, which arise from a common stem/progenitor cell (figure 3). However, horizontal relationship among these MBs is still not clear (Wagers and Conboy, 2005). It is noteworthy that C2C12 MBs were originally isolated from 2-month old adult skeletal muscle, so they are generally considered as a model system for the satellite cell and fetal MB (Yaffe and Saxel, 1977)

Molecular biology of skeletal muscle development (myogenesis)

1.1. Specification of skeletal muscle progenitor / stem cell; role of Pax3 and Pax7

1.1.1. Role of Pax3

The earliest molecule involved in skeletal muscle lineage specification during embryo development is the paired box transcription factor, *paired box gene-3* (*pax3*). Pax3 is a member of the paired box gene family, which is characterized by the presence of a paired domain. Most of the Pax genes encode sequence specific DNA binding transcription factors, which recognize TAAT(N2-3)ATTA motif with some variations. Pax family proteins play a number of roles in tissue and organ development (Chi and Epstein, 2002; Lang et al., 2007; Wilson et al., 1993). In mouse embryo development, Pax3 is expressed in the PSM (E8) and also newly segmented epithelial somites (E10.5-13.5) (Takahashi et al., 2007; Williams and Ordahl, 1994). The mouse embryo lacking Pax3 protein (*pax3*-/-; splotch mouse) fails to form limb muscles (Bober et al., 1994; Borycki et al., 1999b). Although the dermomyotome forms in the *pax3*-/- mouse embryo, the muscle precursor cells from the VLL of the hypaxial dermomyotome do not migrate to the limb (Goulding et al., 1994; Williams and Ordahl, 1994). Therefore, Pax3 is required for limb but not trunk muscle formation.

Furthermore, adenovirus mediated ectopic expression of Pax3 in the lateral mesoderm *in vitro* induces *myogenic differentiation1* (*myod*) and *myogenic factor-5* (*myf5*) expression (Maroto et al., 1997). Compound loss of *pax3* and *myf5* causes loss of body muscle with the exception of the head region in the embryo, so the

myod gene is genetically located down-stream of *pax3* and *myf5* (Tajbakhsh et al., 1997), and *myf5* expression is also regulated by Pax3 in mature somites (Bajard et al., 2006). Therefore, Pax3 regulates the initiation of myogenic gene expression program. Consistent with these observations, vessel-associated stem cells, mesoangioblasts, are capable of differentiating to skeletal muscle in a Pax3 dependent manner (Messina et al., 2009), and also exogenous expression of Pax3 is sufficient for activation of the skeletal muscle differentiation gene expression program in P19 embryonic carcinoma cells (Ridgeway and Skerjanc, 2001). However, myogenic role of Pax3 is context dependent. For example, in the normal course of myogenic differentiation of C2C12 myoblast cell, ectopic expression of Pax3 inhibits myogenesis, and C3H10T1/2 fibroblast cell conversion to myocyte by MyoD but not Myogenin (MyoG) is also interfered with Pax3 expression (Epstein et al., 1995). In adult skeletal muscles, Pax3 expression becomes undetectable with the exception of a small fraction of musculatures, for example the diaphragm and a small fraction of satellite cells (Kassar-Duchossoy et al., 2005; Kuang et al., 2007; Relaix et al., 2004). However, since at the initial stage of satellite cell activation and proliferation, Pax3 expression is transiently up-regulated (Boutet et al., 2007; Conboy and Rando, 2002) (figure 8 and 9), Pax3 may be required for muscle growth and regeneration. However, in agreement with above observations, Pax3 is mono-ubiquitinated and targeted for proteasome mediated degradation (Boutet et al., 2007). Therefore, for adult skeletal muscle growth and regeneration, Pax3 may play a role in activation and early proliferation but need to be eliminated for MB

differentiation. However, more recent study documented that Pax3 is dispensable for adult skeletal muscle growth and regeneration (Lepper et al., 2009).

1.1.2. Role of Pax7

Pax7, another member of the Pax gene family, is expressed in the central region of the dermomyotome (figure 4). The initial phenotypic analysis of the loss of Pax7 did not detect any overt defect in the skeletal muscle (Mansouri et al., 1996). However, a subsequent detailed analysis of the skeletal muscle in the *pax7*-/- mouse identified an almost complete loss of satellite cells (precursor cells for skeletal muscle) but not another classes of muscle derived stem cells leading to a muscle regeneration defect in the postnatal mouse (Seale et al., 2000). The reduction of the number of satellite cells was also observed during fetal muscle growth in the *pax7*-/- mouse, and Pax7 was found to be required for satellite cell survival independent of Pax3 (Kassar-Duchossoy et al., 2005; Relaix et al., 2006). A compound loss of *pax3* and *pax7* causes an arrest in skeletal muscle development after forming the primitive myotome (Relaix et al., 2005). Although some of the roles of Pax3 can be compensated by Pax7 in the dorsal neural tube, neural crest cell, and somite development, Pax3 is essential for migration of muscle progenitor cells to the limb (Relaix et al., 2004), and Pax7 is required for satellite cell survival (Relaix et al., 2006). Therefore, Pax3 and Pax7 play distinctive essential roles in myogenesis. Since the expression pattern of Pax3 and Pax7 is clearly different in muscle progenitor cells in each phase of muscle differentiation (embryo, fetal, and adult myogenesis), it can be used to classify the MB. The embryonic MB is Pax3+/Pax7-,

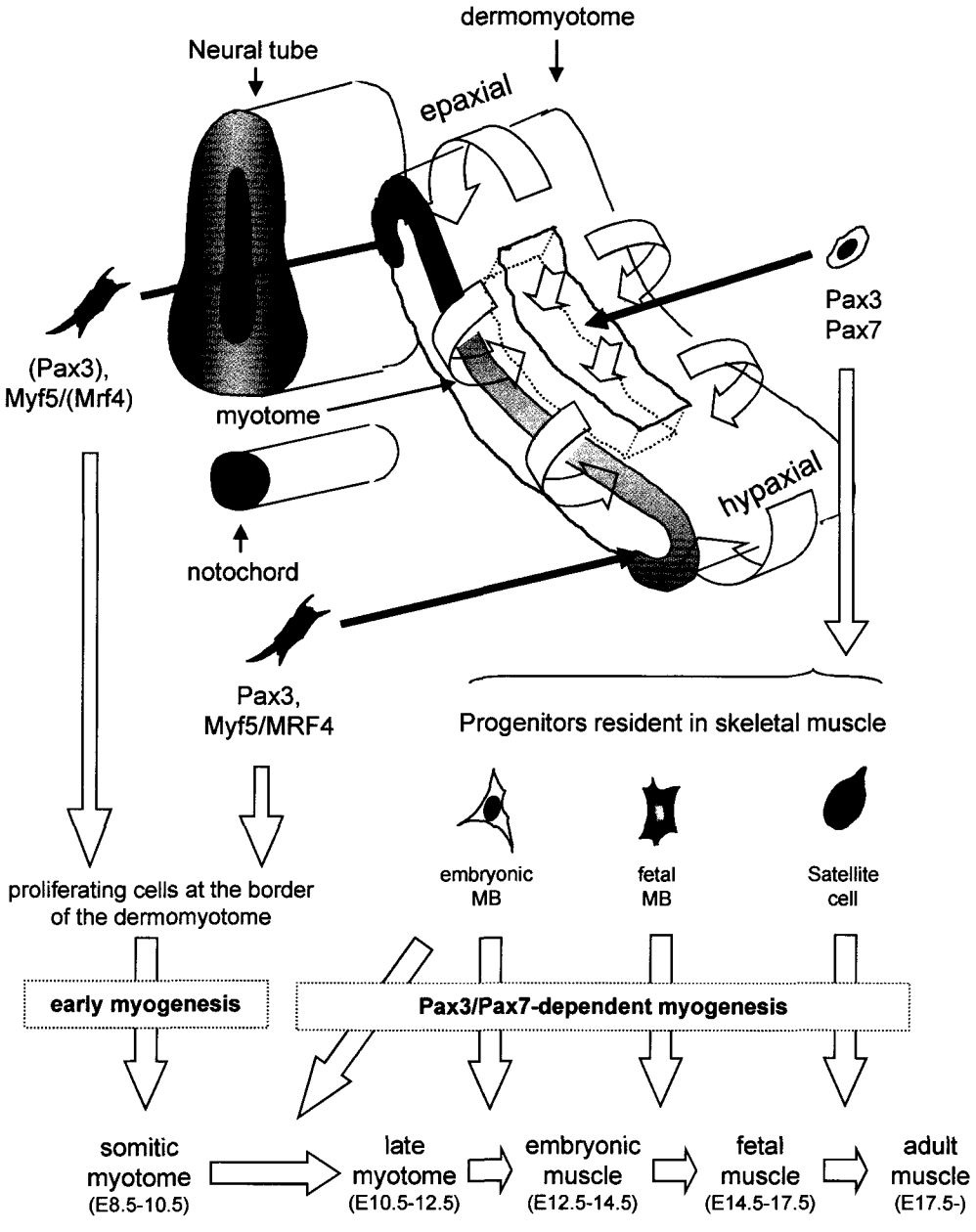

Figure 4. **Anatomical origin of muscle progenitors**

the fetal MB is Pax3+/Pax7+, and the satellite cell (adult MB) is Pax3-/Pax7+ with the exception of some Pax3+/Pax7+ progenitor cells. These differences may explain why each category of MB responds differently to the environmental cues during embryonic development (Hutcheson et al., 2009).

1.2. Specification of skeletal muscle; role of cell-cell communication

The cells in newly formed epithelial somites are specified not only as myotome but also dermatome, sclerotome, and syndetome (figure 2). This specification process is highly dependent on the position of the cells in the somite. Accumulating evidence suggests that the communication with the surrounding axial structures by means of soluble secreted factors or extracellular protein-protein interactions play roles in the specification of the cells during embryo development.

For muscle cell lineage specification, Sonic hedgehog (Shh) protein secreted from the notochord and the floor plate of the neural tube is required for expression of Myf5 in the dorsal medial region of the somite (epaxial muscle precursors) through activation of Gli transcription factors, and Shh is also essential for sclerotome formation in the ventral region of the somite which antagonises dermatome formation (Fan et al., 1995; Gustafsson et al., 2002; Johnson et al., 1994; Munsterberg et al., 1995) (figure 5). Hypaxial muscle formation is regulated by the signals from the lateral plate, dorsal ectoderm, and dorsal neural tube (Cossu et al., 1996; Pourquie et al., 1995; Pourquie et al., 1996; Sporle et al., 1996; Takada et al., 1994). The dorsal neural tube secretes bone morphogenic proteins (BMP) and Wnts, and the somite secretes the BMP antagonist, Noggin, whose expression is induced

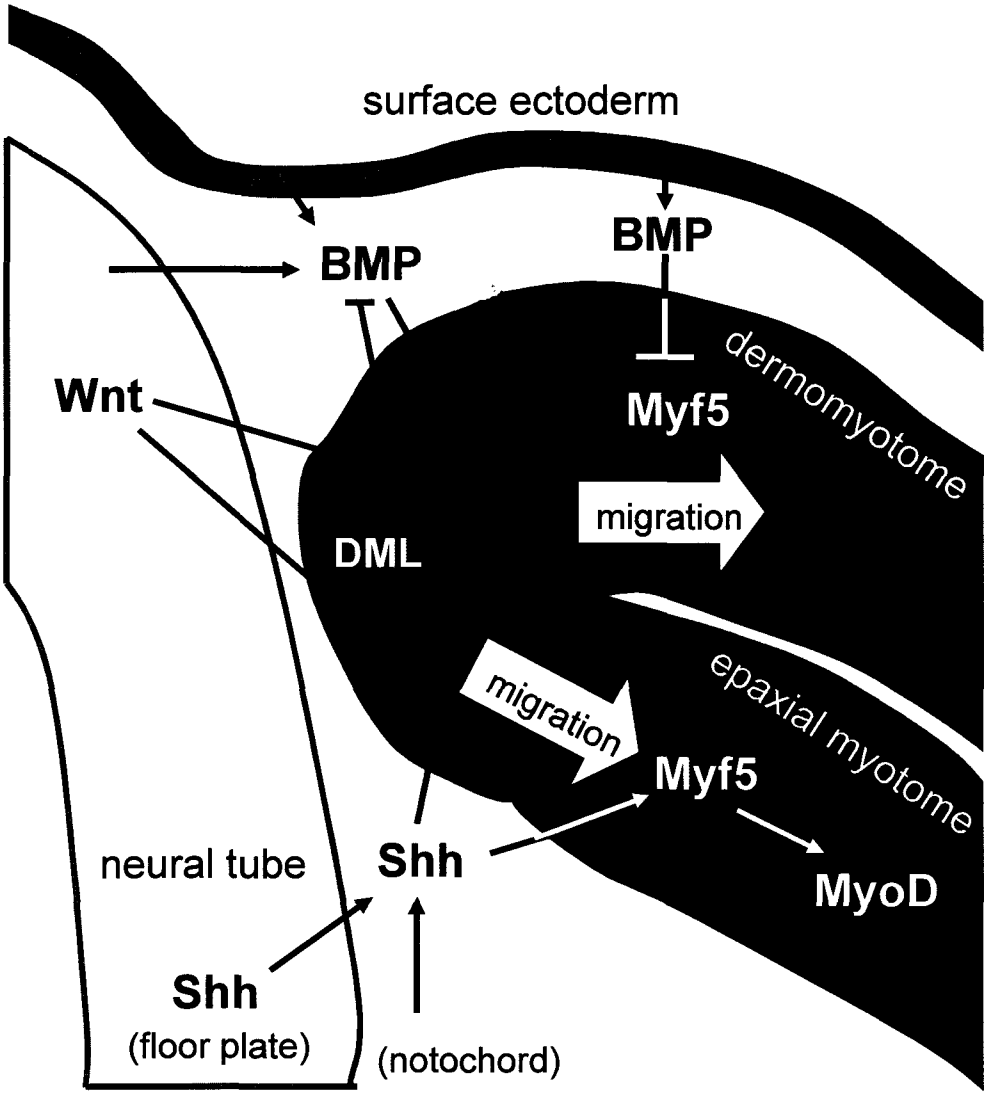

Figure 5. **Myogenic cell specification at the DML**

by the Wnt signalling (Marcelle et al., 1997); the neural tube regulates specification of the cells in the somite in coordination with the dorsal ectoderm and somites (Capdevila et al., 1998; Ikeya and Takada, 1998) (figure 5). The epaxial muscle precursors maintain in an unspecified state in the dermomyotome due to high BMP from the surface ectoderm. However, in the DML, the epaxial muscle precursors are also under the influence of Wnts secreted from the neural tube. The Wnts activate Noggin, which antagonizes the BMP signalling, and also induce Gli transcription factors, which induce *myf5* gene expression. In addition, the ventral region is the 'Shh-rich' environment due to its secretion from the notochord and the floor plate of the neural tube. As a result, proliferation of cells at the DML contribute to the growth of the dermomyotome on the dorsal side ('re-unspecified' by BMP), and on the ventral side, the epaxial muscle precursors maintain *myf5* expression and produce Myf5 proteins leading to the expression of MyoD and differentiation into myocytes and formation of the epaxial myotome (figure 5).

2.1. Differentiation of skeletal muscle; role of myogenic regulatory factor (MRF)

For the induction of the skeletal muscle differentiation program, myogenic regulatory factors (MRFs) are essential. The MRFs are members of the basic helix-loop-helix (bHLH) family of sequence specific DNA binding transcription factors. The MRFs consist of Myogenic Differentiation-1 (MyoD), Myogenic Factor-5 (Myf5), Myogenin (MyoG), and Myogenic Regulatory Factor-4 (MRF4/Herculin/Mfy6) (Pownall et al., 2002) (figure 6A). Most skeletal muscle specific genes have E-boxes (CANNTG) in their regulatory region, and this *cis*-

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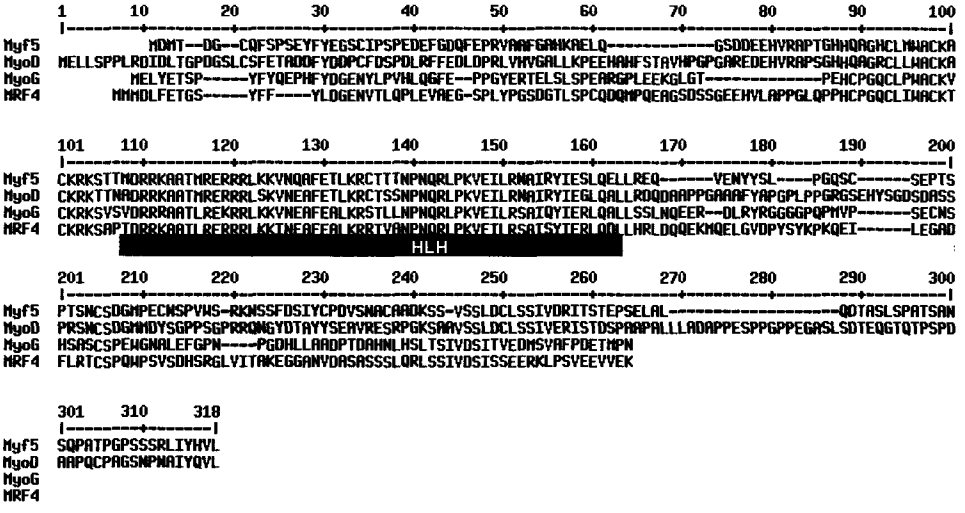

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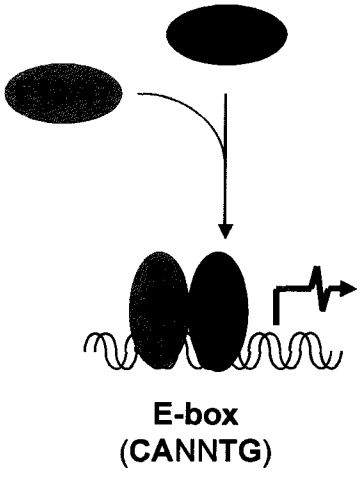

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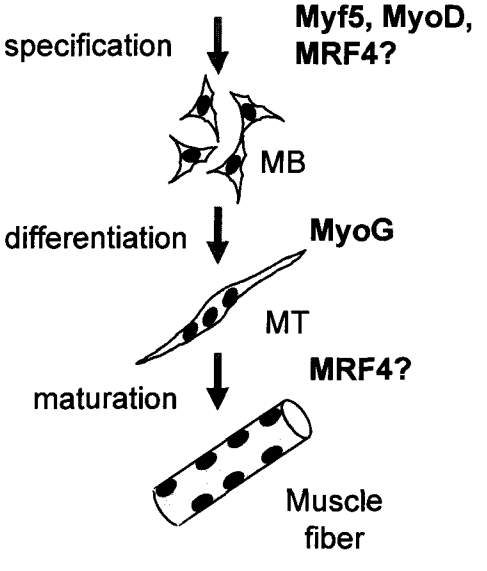


Figure 6. **Myogenic regulatory factor** A) Amino acid sequence alignment of MRFs. B) DNA binding requirements of MRF C) Role of MRF during myogenesis

regulatory element is required for muscle specific expression. The MRFs heterodimerize with ubiquitously expressed E-protein (E12, E47) and bind the E-box found in the regulatory region of muscle specific genes to induce gene expression in co-operation with other transcription factors (Etzioni et al., 2005; Murre et al., 1989) (figure 6B). This leads to the differentiation of muscle progenitor cells to morphologically and biochemically distinctive skeletal myocytes (Olson et al., 1991) (figure 6). Indeed, MyoD and also three other MRFs, if ectopically expressed individually, can convert fibroblasts into myocytes (Davis et al., 1987; Molkentin and Olson, 1996).

Gene targeting studies in a mouse in which a specific MRF or combinations of the MRFs have been genetically ablated demonstrates the MRF's functions *in vivo*. The loss of either *myod* (Rudnicki et al., 1992) or *myf5* (Braun et al., 1992) has no effect on specification of myogenic lineage during embryo development. However, the compound loss of functional *myod* and *myf5* causes loss of MB and muscle fibers without affecting mesenchymal multi-potentiality (Kassar-Duchossoy et al., 2004). Therefore, for cell lineage specification, either Myf5 or MyoD is required, and both work as lineage determination factors. A targeted mutation of the *myog* gene causes loss of most of the skeletal muscle fibers; yet, the MB population was expanded. Therefore, the skeletal muscle lineage is specified during embryo development but muscle progenitor cell differentiation was interfered with the loss of *myog* (Hasty et al., 1993). Thus, MyoG is essential for the skeletal muscle differentiation. Gene targeting of *mrf4* in mouse did not generate a consistent phenotype (Patapoutian et al., 1995; Zhang et al., 1995) since *mrf4* and *myf5* are genetically linked (Yoon et

al., 1997) (figure 7). MRF4 can specify cell lineage to skeletal muscle progenitor in a double-null mutation of *myf5* and *myod* loci if the mutation of *myf5* locus does not affect on *mrf4* expression in the mouse (Kassar-Duchossoy et al., 2004) and zebrafish embryo (Schnapp et al., 2009). Therefore, the function of MRF4 needs to be elucidated further.

2.1.1. Regulation of the myf5 gene during embryo development

The first MRF expressed during embryonic development is Myf5 which becomes detectable at E8.0 in the DML. The *myf5* gene is induced at E9.5 in the ventral dermomyotome and branchial arches (BAs) (Ott et al., 1991; Summerbell et al., 2000).

Since the transcriptional regulatory region of the *myf5* gene spans more than 140 kb from the transcriptional start site (TSS), and the *myf5* and *mrf4* genes are genetically linked (figure 7), the analysis of *myf5* gene regulation has been a challenging task (Carvajal et al., 2008). Since all skeletal muscle precursor cells (Pax3+/Pax7+) originate from the central region of the dermomyotome with the exception of the somitic myotome, which is developed from the epaxial (DML) and hypaxial (VLL) dermomyotome, the early myogenesis, in which somitic myotome forms (E8.5-10.5) from the DML and VLL progenitor cells, is Pax3/Pax7 independent. Therefore, *myf5* is induced in a Pax3/Pax7 independent manner in the DML, VLL, and BA. The early epaxial enhancer of *myf5* gene is activated by the Shh/Gli, and Wnt/β-catenin pathways, and Wnt mediated Noggin indirectly

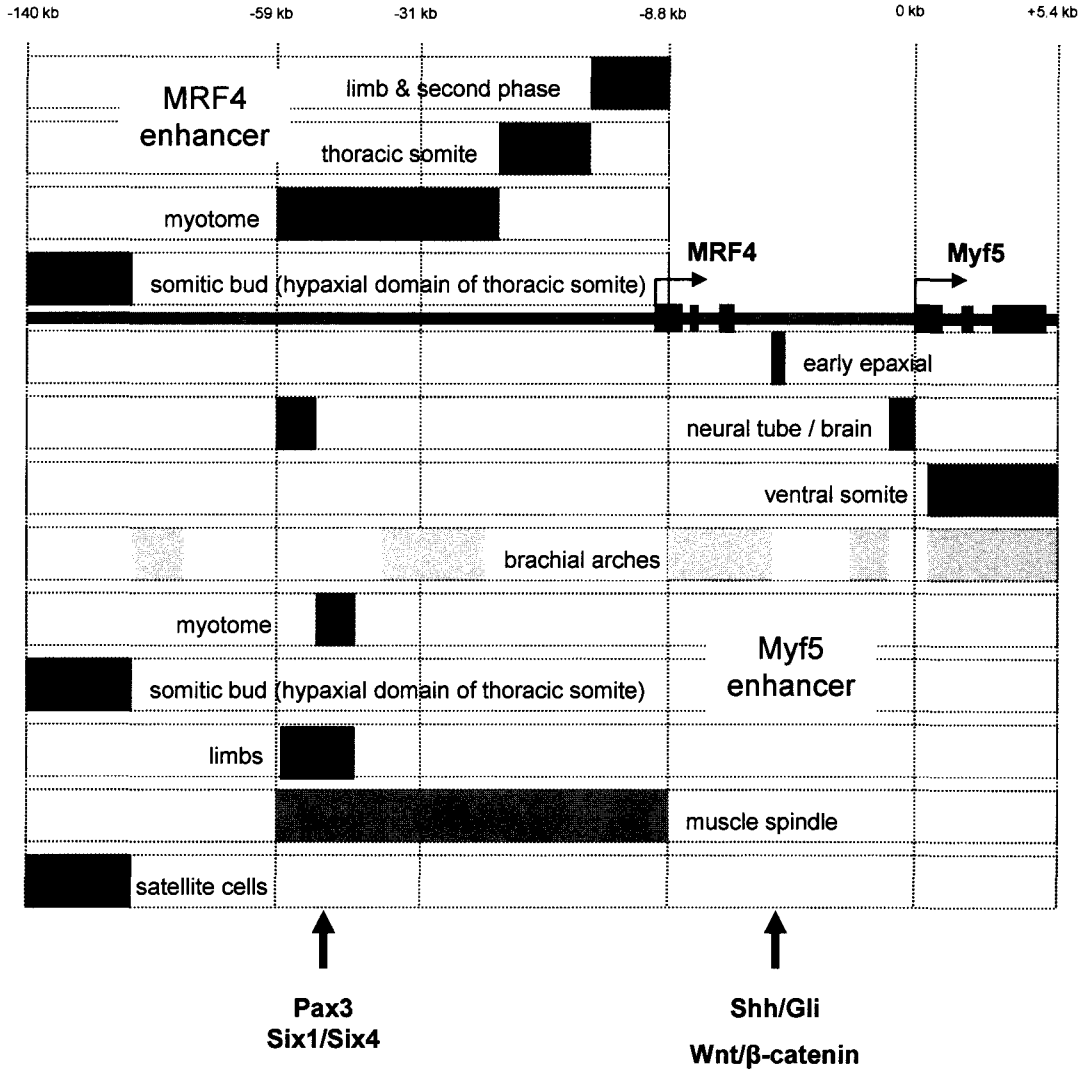


Figure 7. **Enhancer/promoter region of *mrf4/myf5* locus**

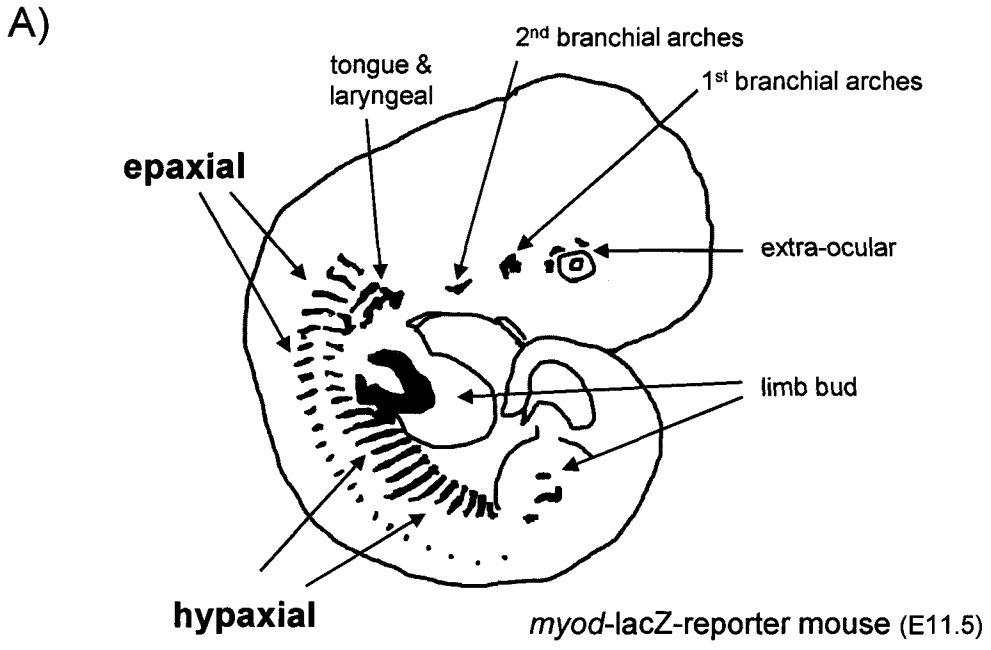
antagonizes the negative regulatory BMP signalling (Borello et al., 2006; Gustafsson et al., 2002; Hadchouel et al., 2000) (figure 5 and 7). However, the other group disputes this observation (Teboul et al., 2003). On the other hand, for the Pax3 dependent induction of the *myf5* gene, the enhancer element located at -58/-48 kb of the TSS of *myf5* gene is required, and removal of this region caused the loss of the activity of the *myf5* promoter and expression of *myf5* gene. It further leads to loss of limb muscles (Hadchouel et al., 2000). In the -58/-56 kb, a putative paired domain-binding site was identified, and this region is required and sufficient for *myf5* expression in the muscle precursor cells in limbs and particularly somites. Pax3 can bind and activate the *myf5* promoter through this putative binding site (Buchberger et al., 2007) (figure 7). Furthermore, Six1 and Six4, genetically located up-stream of the *pax3* gene, bind a 145 bp-element located at the -57.5 kb region of this enhancer, and together with Pax3, they activate the *myf5* gene expression in the limbs and mature somites (Giordani et al., 2007) (figure 7). This is a complete agreement with the defect in skeletal muscle development in the *six1* and *six4* compound 'knock-out' mouse (Grifone et al., 2005). An another transcription factor regulates the *myf5* expression during embryo development is T BoX family member-6 (Tbx6), which is important regulator of somite specification (Tatsumi et al., 2006). BMP/Smad6 signalling co-operates with Smad specific E3 ubiquitin protein ligase 1 (Smurf1), an E3 ubiquitin ligase, targets Tbx6 for degradation. As a result, the BMP signalling inhibits *myf5* expression (myotome specification) through degradation of Tbx6 mediated by Smad6/Smurf1 (Chen et al., 2009b). However, this needs to be verified in vivo.

In adult skeletal muscle, *myf5* expression localizes to the muscle fiber associated satellite cell and muscle spindle, which is a distinctively small muscle fiber in diameter and works as a type of stretch-sensitive mechano-receptor (Passatore et al., 1983). Surprisingly, *myf5* expression in the satellite cell and the muscle spindle are driven by the -140/-88 kb and -59/-8.8 kb of the regulatory region of *myf5* gene respectively (Zammit et al., 2004a).The *myf5* gene enhancer/promoter-reporter gene can be activated in the neural tube during embryo development, but Myf5 protein is not detectable in the corresponding tissue (Daubas et al., 2000). Therefore, transcriptional induction of the *myf5* gene is regulated by a variety of transcription factors in a developmental stage and cell specific manner (figure 7). In addition, a very recent study suggests that *myf5/mrf4* gene expression is transcriptionally regulated by 'transcription balancing sequences', which regulate enhancer-promoter interaction for productive transcription (Carvajal et al., 2008). The *myf5* gene expression is maintained during the fusion of MBs, but starts down-regulated during late gastrulation, and only remains in satellite cells and muscle spindles in the adults (Zammit et al., 2004a).

2.1.2. Regulation of the myod gene during embryo development

Following Myf5, MyoD begins to express at E10.5 in the myotome (Chen et al., 2001) (figure 5). In the *myf5*-/- embryo, MyoD expression is delayed but is not abolished in the DML and VLL. However, the *pax3*-/- and *myf5*-/- double null embryo fails to form skeletal muscle with the exception of the head region. Therefore, either Pax3 or Myf5 is required for *myod* expression during embryonic

development (Tajbakhsh et al., 1997). The functional cis-regulatory regions of *myod* promoter/enhancer was isolated (Goldhamer et al., 1992). A 256 bp element (core enhancer sequence; CES) located at -20 kb of the TSS of the mouse *myod* gene sufficiently reproduces the endogenous gene expression pattern (Goldhamer et al., 1995) (figure 8B). In addition to the CES, at -5 kb of the TSS of the *myod* gene has the distal regulatory region (DRR), and the CES supports spatiotemporal expression pattern of endogenous *myod* gene if introduced into embryo as a heterologous reporter except with temporal defects in the hypaxial myotome and limb buds (Chen et al., 2001) (figure 8A). Even though *myod* gene is genetically located down-stream of *myf5* and *pax3*, regulation of both the DRR and the CES is not directly regulated by Myf5 and Pax3 (Chen and Goldhamer, 2004; Chen et al., 2002). Search of the CES regulatory factor by a yeast one-hybrid screen for factors that interact with the *myod* core enhancer DNA identified a novel C2H2 zinc finger protein, glucocorticoid-induced gene-1 (Gig1), whose expression pattern overlaps with *myod* expression in mouse embryo (Yamamoto et al., 2007). However, a molecular mechanism by which Myf5 and Pax3 induce *myod* expression during embryonic myogenesis has not been characterized yet. A very recent study showed that for injured adult skeletal muscle regeneration, which is defective in *myod*-/- mouse (Asakura et al., 2007), Pax3 and forkhead box O3 (FoxO3) are required for up-regulation of *myod* gene, and they bind the cis-regulatory elements located between the DRR and CES of the *myod* gene directly (Huang et al., 2008) (figure 8B).



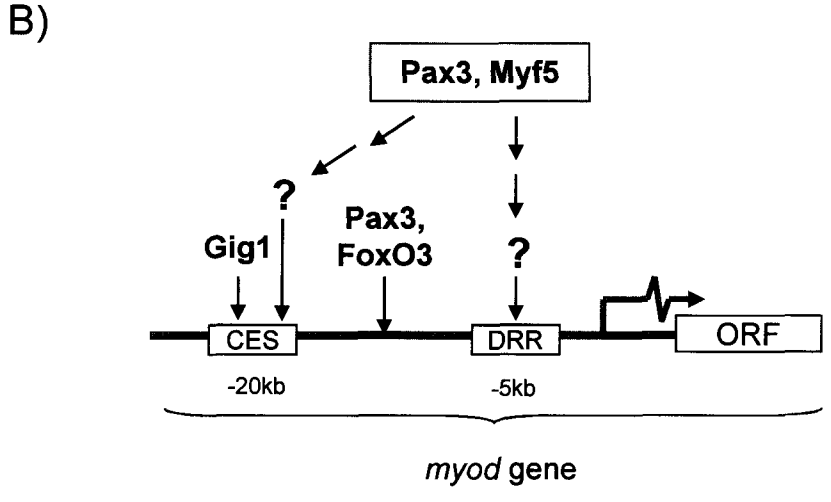


Figure 8. **MyoD expression** A) *myod* Core Enhancer (CES) –*LucZ* expression. B) transcriptional regulation of *myod* gene.

2.1.3. Regulation of the mrf4 gene during embryo development

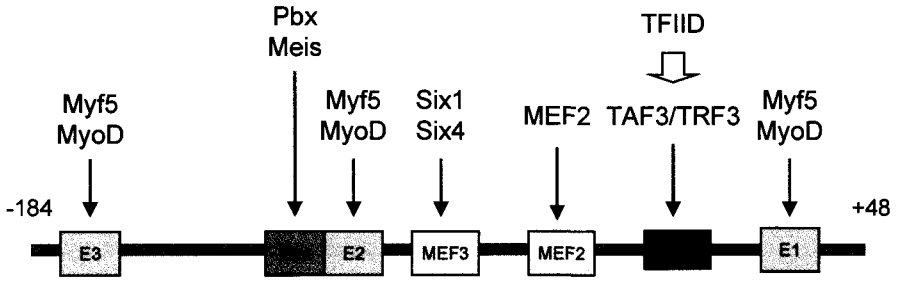
MRF4 expression is detected at E8.0 in the undifferentiated cells in the hypaxial domain of thoracic somites (the somitic bud) (Summerbell et al., 2002), and is activated at E9.0 in the central myotome of rostal somite. With exception of limbs and branchial somites, all somites express *mrf4* gene by E10.0 and again down regulated by E12.5, and then the third phase of activation, in which the expression is sustained in adult skeletal muscles (Carvajal et al., 2001). As discussed above, although *mrf4* expression becomes transiently detectable in the somite myotome during embryogenesis, *mrf4* expression is up-regulated during late fetal development to eventually become the predominant myogenic bHLH factor expressed in adult skeletal muscle. Therefore, it has been proposed that MRF4 may play role in skeletal muscle maturation. In *mrf4* null embryo, MyoG was greatly up-regulated, and it was suspected the compensatory mechanism (Zhang et al., 1995). This notion was also supported by the fact that the loss of MyoG can be compensated by *mrf4* expression driven by *myog* promoter in embryo skeletal muscle development (Zhu and Miller, 1997). However, more recent observations indicate that MEF4 may function as a skeletal muscle lineage determination factor (Kassar-Duchossoy et al., 2004). Since *myf5* and *mrf4* are genetically linked, the loss of mutation on *mrf4* locus also affects *myf5* expression (Carvajal et al., 2008; Yoon et al., 1997) (figure 7). A new *myf5*-/- and *myod*-/- double null embryo, in which the *mrf4* gene expression is not affected by the manipulation, surprisingly did

not show severe skeletal muscle defect phenotype observed in the early *myf5*-/- and *myod*-/- double "knock-out" embryo (Valdez et al., 2000) suggesting that MRF4 and Myf5 are genetically located up-stream of MyoD (Kassar-Duchossoy et al., 2004). In zebrafish skeletal muscle development, early expression of MRF4 but not MyoG can reverses the defective skeletal muscle phenotype of the *myf5*-/- and *myod*-/- doubly targeted embryo further supporting the lineage specification role of MRF4 (Schnapp et al., 2009).

2.1.4. Regulation of the myog gene during embryo development

The loss of MyoG cannot be compensated for by the other MRFs, so MyoG is a unique MRF and essential for skeletal muscle differentiation during embryo development (Hasty et al., 1993). MRFs and Myocyte enhancer factor 2 (MEF2) play important roles in the *myog* gene regulation during embryo development. The 5'regulatory region of the *myog* gene has MRF binding sites (E-box) and MEF2 binding site (figure 9), and the mutation of these sites attenuates the *myog* expression in the embryo (Cheng et al., 1993; Yee and Rigby, 1993). In *myog*-/- embryo, muscle lineage specification occurs normally (formation of muscle progenitors), but they could not differentiate into multinucleated myotubes (Nabeshima et al., 1993). In agreement with these observations, loss of MyoG does not affect *myod* expression and primary myogenesis (MB formation) (Venuti et al., 1995). A -184/+18 bp *myog* promoter respect to the TSS can reproduce the *myog* expression pattern during embryo development (Cheng et al., 1993), and evolutionarily conserved MEF3 sites (TCAGGTT) are required for *myog*

A)



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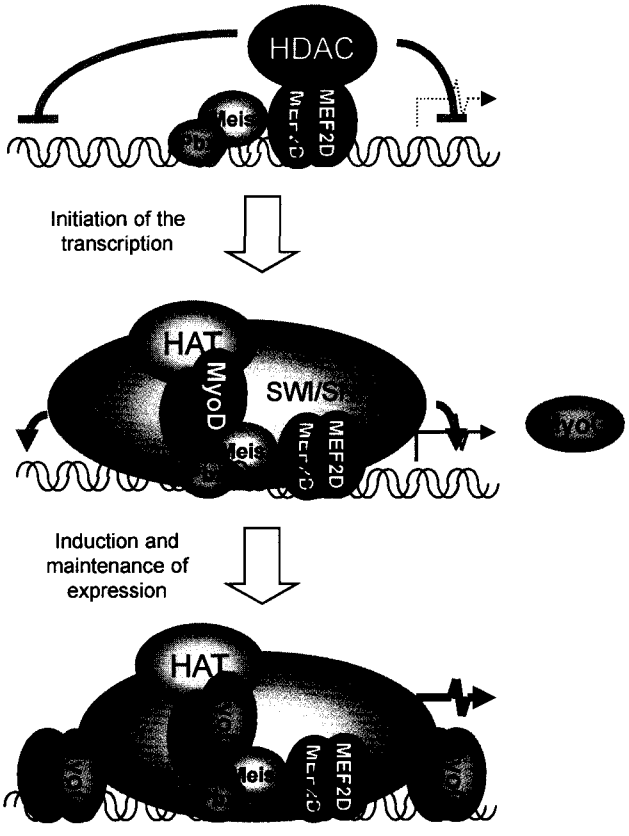


Figure 9. **Transcriptional regulation of the *myog* gene** A) *cis*-regulatory elements and transcriptional regulators of the *myog* gene. B) transcriptional regulator complex formation on the *myog* gene.

expression. The MEF3 sites are recognized and trans-activated by the homeoproteins of the Six/sine oculis family, Six1 and Six4 (Spitz et al., 1998). Although *six4-/-* embryos bone normally without any gross defects (Ozaki et al., 2001), *six1-/-* and *six4-/-* compound mutant embryos have a skeletal muscle defect (Grifone et al., 2005). Activation of the *myog* gene by MyoG in co-operation with MEF2D is required for full activation of the *myog* gene expression. MEF2D and MyoG, but not MyoD, co-recruit SWI/SNF complex containing an ATP-dependent chromatin remodelling enzyme, BRG-1, which alters chromatin structure at the regulatory region of the *myog* gene for the terminal differentiation (Ohkawa et al., 2006; Ohkawa et al., 2007) (figure 9). It is also reported that for the MyoD dependent *myog* gene activation, TAF3/TRF3 complex replaces TFIID and directs the transcriptional initiation of the *myog* gene transcription (Deato et al., 2008). Therefore, chromatin remodelling and general transcription factor switch are also important regulatory steps to induce the *myog* gene.

2.2. Differentiation of skeletal muscle; role of Myocyte enhancer factor-2 (MEF2)

Myocyte enhancer factor-2 (MEF2) was isolated from C2C12 myotubes and BC3H1 myocytes as a trans-activator on the enhancer element in the muscle creatine kinase gene (Gossett et al., 1989). *mef2* genes are taxonomically part of the MADS-box gene super-family that encode DNA binding proteins involved in yeast mating type decisions (MCM1), plant development (Agamous and Deficiens), and serum responsivity of mammalian cells (Serum Response Factor: SRF) (Jarvis et

al., 1989; Norman et al., 1988; Sommer et al., 1990; Yanofsky et al., 1990). There are four *mef2* genes (*mef2a, -b, -c, and –d*) in mammals, and they generate a few isoforms from each gene. MEF2 protein forms homo- or heterodimers and binds to dsDNA in a sequence specific manner (the consensus sequence of the MEF2 site; (C/T)TA(A/T)$_4$TA(G/A)) (Pollock and Treisman, 1991). Although MEF2 expression is not restricted in skeletal muscle (Potthoff and Olson, 2007), its activity is highly localized in muscle and neural cell lineages during development of mouse embryo (Naya et al., 1999). In myogenic lineage, *mef2c* is expressed at E9.0 in rostral myotome after *myf5* and *myog* expression. At E9.5, expression of *mef2a, -c,* and *–d* genes are detected in myotome. After E12.5, *mef2* is expressed in a wide variety of tissues (Edmondson et al., 1994). This indicates MEF2 activity is post-transcriptionally or –translationally regulated. Indeed, MEF2 activity is extensively regulated by variety of post-translational modification (Potthoff and Olson, 2007). DNA binding activity of the MEF2 dimers seems not to be regulated in a cell type specific manner (Ornatsky and McDermott, 1996). We and other groups documented that MEF2 transcriptional activity is regulated by phosphorylation coupled recruitment of either histone deacetylases (HDACs)/co-repressors or Histone acetyltransferase (HAT) enzymes/co-activators (Du et al., 2008; Gordon et al., 2009; Perry et al., 2009; Potthoff and Olson, 2007; Zhang et al., 2007b) (figure 10A). More importantly, most muscle specific genes have a conserved E-box for MRF binding and MEF2 site in their regulatory region, and MEF2 and MRF co-operates synergistically for the up-regulation of these genes in muscle cells (Black and Olson, 1998; Olson et al., 1995) (figure 10B).

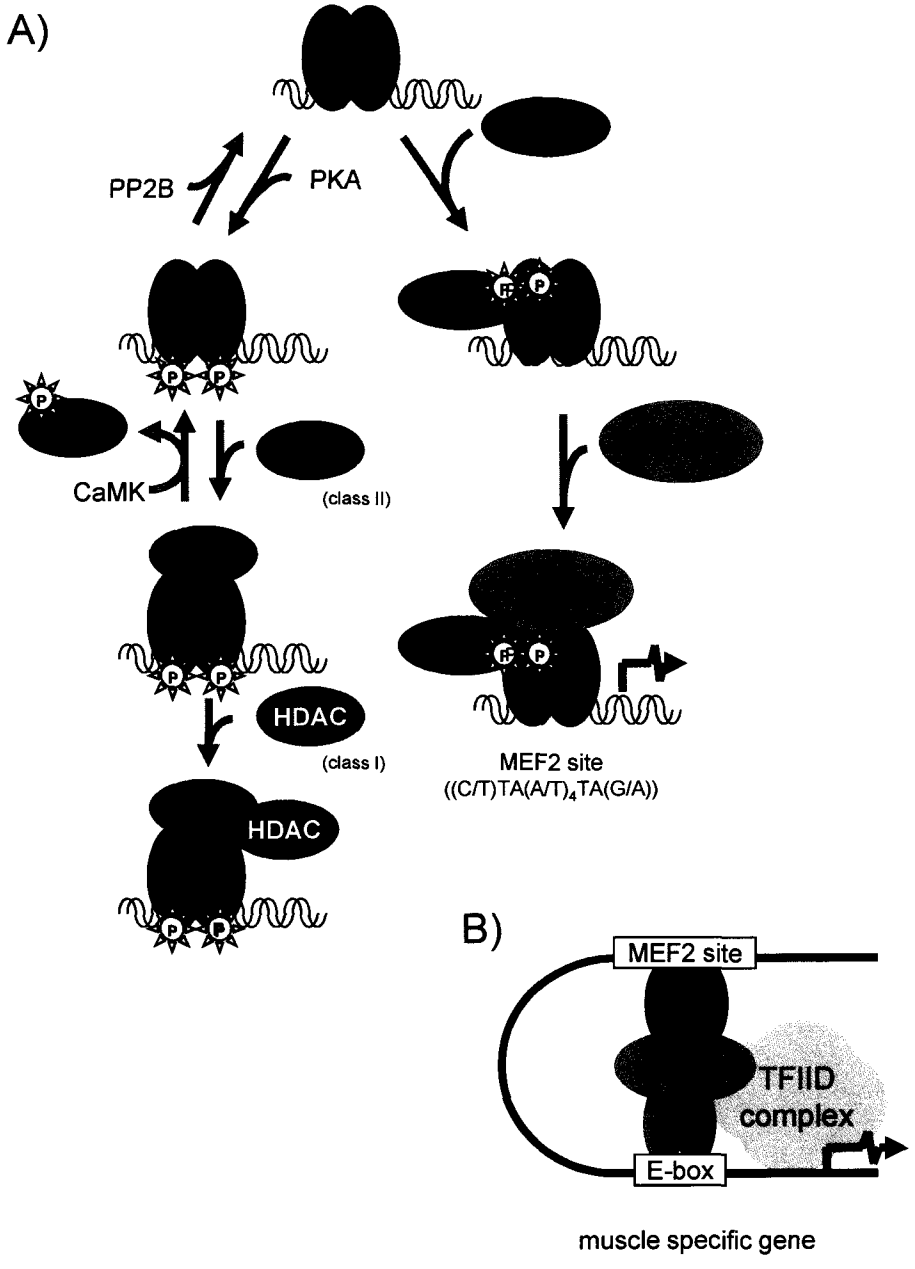


Figure 10. **MEF2 regulation** A) Phosphorylation coupled HDAC/HAT recruitment model. B) Co-operation between MEF2 and MRF on muscle specific promoter.

2.3. Differentiation of skeletal muscle; role of signalling molecules

Primary myogenesis occurs at E10.5-12.5, in which only the fraction of MB population, embryo MBs, differentiates to form primary fibers. The remaining MBs (fetal MBs; Pax3+/Pax7+) maintain their committed state (Pax3+/Pax7+) but somehow avoid differentiation till E14.5-17.5 (Kassar-Duchossoy et al., 2005; Relaix et al., 2006; Relaix et al., 2005). At E14.5-17.5, secondary myogenesis takes place in which the fetal MBs fuse to form secondary myofibers (initially smaller in diameter) around the primary myofibers (Kelly and Zacks, 1969). The Fetal MBs may also fuse to the primary myofibers (Zhang and McLennan, 1995) (figure 3 and 4). After formation of fetal muscle (E16.5), mono-nucleated satellite cells can be identified between the plasma membrane and the basal lamina of the myofibers (Mauro, 1961) (figure 11).

It has been established that these MBs (embryo MB, fetal MB, and satellite cell (adult MB)) have specific characteristics and respond to extracellular cues in a distinct manner (Biressi et al., 2007a; Biressi et al., 2007b; Cossu and Biressi, 2005; Cossu et al., 1987; Cossu et al., 1988; Cossu and Tajbakhsh, 2007; Cusella-De Angelis et al., 1994; Hutcheson et al., 2009; Zappelli et al., 1996).

2.3.1. Roles of BMP signalling

BMP signalling controls specification of myogenic lineage. Interestingly, in the presence of BMP4 (50ng/ml), embryo MBs isolated from the somites and limbs of E11.5 embryo, but not fetal MBs from E16.5, can differentiate into muscle myosin

heavy chain (MyHC)-positive multinucleated myotubes *in vitro* (Biressi et al., 2007b). Genome-wide gene expression analysis of the embryo MBs and fetal MBs revealed that key genes in the BMP/TGFβ signalling are differentially expressed. For example, *myostatin, smad6, smad7, mef2c, fgf, follistatin,* and *connective tissue growth factor (ctgf)* are higher, and *pax3, junb, collagens*, and *type II tgfβreceptor* are lower in the embryo MBs (Biressi et al., 2007b). In addition, a potent inhibitor for myogenic differentiation of the fetal MBs, TGFβ (10ng/ml), also failed to inhibit the embryo MB's myogenesis *in vitro* (Biressi et al., 2007b). Therefore, although the molecular mechanisms are not still clear, these genes that are differentially regulated in embryo and fetal MB might be targets of the BMP/TGFβ signalling and they are potentially important genes for myogenic differentiation.

2.3.2. Roles of Shh signalling

Sonic hedgehog (Shh) is secreted from notochord and the floor plate of the neural tube at critical times for somite specification (figure 5), and the roles of Shh were originally characterized as an enhancer of the formation of sclerotome and a suppressor of the dermatome formation (Johnson et al., 1994). The myotome formation mediated by induction of MRF may be directed by the combinatorial activity of the Shh and Wnt (Munsterberg et al., 1995), and one of the mechanisms by which the Shh inducing *myf5* expression is co-operative up-regulation of a BMP4 antagonist Noggin to relieve BMP4's repressive effect on the *myf5* expression (Borycki et al., 1999a; Hirsinger et al., 1997; Marcelle et al., 1997) (figure 5). Noggin induction is essential for the DML specification for sclerotome

and epaxial myotome through blocking BMP4 signalling (McMahon et al., 1998). The Shh signalling also regulates cell lineage specification in the epaxial myotome through the *myf5* gene up-regulation through the epaxial somite enhancer of the *myf5* gene by Gli transcription factors (Gustafsson et al., 2002) (figure 5). Gli2 and -3 are also required for sclerotomal gene induction by Shh (Buttitta et al., 2003). *Myod* expression in the somite is also regulated by Shh mediated by Gli2/4 (Borycki et al., 1998). Shh maintains but not induces *myod* expression in the somites (Coutelle et al., 2001; Marcelle et al., 1999). Shh also induces expression of the cell surface heparan sulfate proteoglycans, which regulate heparan-dependent Wnt signalling is required for *myod* expression in C2C12 cells (Dhoot et al., 2001).

2.3.4. Roles of Wnt signalling

Another signalling molecule known for embryo myogenesis regulator is Wnt (a secreted glycoprotein), which is secreted from the neural tube (Wnt1) (figure 5). Wnt induces expression of Noggin, which is a secreted protein and binds and inactivates BMP4 (an antagonist of BMP4), at the DML of epaxial dermomyotome (Hirsinger et al., 1997). Since BMP indirectly regulates Wnts expression, at the DML the dorsal portion of somite patterning is determined by the BMP and Wnts from the dorsal neural tube, the Shh from the floor plate of the neural tube and notochord, and Noggin in the somite (Marcelle et al., 1997; Reshef et al., 1998) (figure 5).

Wnt/β-catenin 'canonical' pathway is required for induction of the *myf5* gene in the epaxial domain of the somite, where activated T-cell specific transcription

factor/lymphoid enhancer-binding factor (Tcf/Lef) by the 'canonical' Wnt signalling binds the early epaxial enhancer of the *myf5* gene and further co-operates with Shh/Gli for full induction for the *myf5* gene (Borello et al., 2006) (figure 5). Wnts also induce cyclic AMP responsive element binding protein (CREB) through activation of the protein kinase A (PKA) signalling. The CREB is also required for Wnt inducing *pax3, myf5,* and *myod* expression during embryo myogenesis (Chen et al., 2005). Furthermore, the β-catenin independent Wnt signalling pathway (non-'canonical' Wnt signalling) activates the *myod* gene expression in a protein kinase C (PKC) activity dependent manner, in which PKC activates the Pax3 transcriptional properties and Pax3 induces the *myod* gene expression (Brunelli et al., 2007). Wnts are also important for the adult skeletal muscle growth and injury repair (Le Grand et al., 2009; Otto et al., 2008).

Growth and regeneration of adult skeletal muscle

A major goal for the developmental biology is to reveal the mechanism by which an elegantly organized collection of multiple tissues and organs is generated from a single cell during embryo development. Adult stem cells must have an ability to produce both types of progenys; one for self-renewal to maintain 'stem' population and the other for growth or regeneration of given specialized cells to maintain growth and regeneration capacity. Skeletal muscle satellite cells on the adult muscle fibers are now considered as adult stem cells, and these satellite cells provide the life-long growth and regeneration capabilities for the skeletal muscles (Collins et al., 2005; Kuang et al., 2008; Seale and Rudnicki, 2000).

1.1. Satellite cell marker; Myf5 and Pax7

1.1.1. Role of Myf5

It has been established that adult myogenesis differs from embryo and fetal myogenesis at the molecular level. The most obvious differences are roles of MyoD and Pax7. In embryo and fetal myogenesis, loss of MyoD can be compensated by Myf5 with exception of muscle development in head region (Rudnicki et al., 1992). However, for adult skeletal muscle regeneration, MyoD is essential (Asakura et al., 2007). These observations suggest that Myf5 could not replace at least some of the MyoD's functions in the adult satellite cell differentiation but not embryo and fetal myogenesis. Interestingly, the *myf5*-/- MBs proliferate at much lower rate than normal MBs. In contrast, the *myod*-/- MBs grow faster and express higher level of Myf5 than the normal MB *in vitro* (Cornelison et al., 2000; Sabourin et al., 1999; Yablonka-Reuveni et al., 1999). Although MyoD is much more efficient than Myf5 for the induction of muscle specific genes, a set of MyoD and Myf5 target genes are very similar in a microarray analysis in C2C12 cells (a satellite cell derived MB cell line) (Ishibashi et al., 2005). Although non-redundant specific down-stream target genes of MyoD and Myf5 in the satellite cell have not yet identified, it has been suggested that Myf5 plays a role in satellite cell proliferation and that MyoD is required for the growth arrest and differentiation (Asakura et al., 2007; Ishibashi et al., 2005). Since Myf5 expression has been down-regulated in the myonuclei in mature terminally differentiated post-mitotic myofibers, the Myf5 expression is one of the markers for the quiescent satellite cells on the myofibers. However, 10% of

Pax7+ quiescent satellite cells do not express Myf5, so these satellite cells (Pax7+, Myf5-) are not yet committed to the myogenic lineage (Kuang et al., 2007).

1.1.2. Role of Pax7

The other satellite cell marker is Pax7, which is essential for maintenance/survival of satellite cells during embryo development (Seale et al., 2000) but dispensable for embryo and fetal myogenesis (Mansouri et al., 1996) and adult regeneration and muscle growth (Lepper et al., 2009). Specifically, in the *pax7*-/- embryo, primary and secondary myofibers are formed normally, but the satellite cell population is declining progressively due to apoptosis and at birth almost complete loss of the satellite cells on the myofibers causing defects in the skeletal muscle growth and regeneration (Oustanina et al., 2004; Relaix et al., 2006). Pax7 may also be required for the maintenance of the undifferentiated state of the satellite cells by preventing precocious differentiation (Olguin et al., 2007) and of the proliferative capacity by antagonizing MyoD's activity (Zammit et al., 2006). In support of this notion, in chicken, only Pax7+ MB but not Pax7- MB proliferate (Day et al., 2009).
Pax7 expression is inhibited by Myostatin (MSTN), which inhibits satellite cell activation (proliferation) and self-renewal (McCroskery et al., 2003), through activation of Extracellular signal-regulated kinase (ERK) signalling (McFarlane et al., 2008). Interestingly, a microarray and Chromatin immuno-precipitation (ChIP) analysis identified that Pax7 is genetically located at up-stream of the *myf5* gene, and Pax7 directly up-regulates the *myf5* gene expression by recruiting a histone methyltransferase complex in the MBs derived from satellite cells (McKinnell et al.,

2008). Therefore, these observations suggest that Pax7 may be required for maintaining the satellite cell population by supporting *myf5* expression and suppressing *myog* expression induced by MyoD (Olguin et al., 2007). Interestingly, Pax7 and Pax3 become dispensable not only for muscle regeneration but survival of the satellite cell in the adult muscle fiber after 21 days of birth in mouse (Lepper et al., 2009).

2.1. Adult myogenesis; satellite cells to myocytes

Satellite cells are derived from embryonic progenitors (Pax3+, Pax7+, Myf5-, and MyoD-). During fetal myogenesis, these progenitor cells either up-regulate Myf5/MyoD and differentiate into embryonic and fetal muscle or maintain Pax3+/Pax7+/Myf5-/MyoD- state and become satellite cells (Relaix et al., 2005) (figure 3 and 4). Once the satellite cells establish their niche in between the basal lamina and the plasma membrane of the myofibers, most satellite cells induce the *myf5* and down-regulate *pax3* gene expression (Pax7+, Pax3-, Myf5+, and MyoD-) (figure 11). A small population of the satellite cells maintains Pax3 expression (Pax7+, Pax3+, Myf5+, and MyoD-) (Kuang and Rudnicki, 2008). Although most of the satellite cells are expressing Myf5 (Pax7+, Myf5+), a small portion of the satellite cells are not expressing Myf5 (Pax7+, Myf5-), which are an uncommitted population for the myogenic lineage (Kuang et al., 2007) (figure 12). Once activated, these satellite cells (Pax7+, Myf5-) produce progeny, which are ether for self-renewal (Pax7+, Myf5-) or committed (Pax7+, Myf5+) by symmetrical or asymmetrical cell division, respectively (Kuang et al., 2007). Activated and

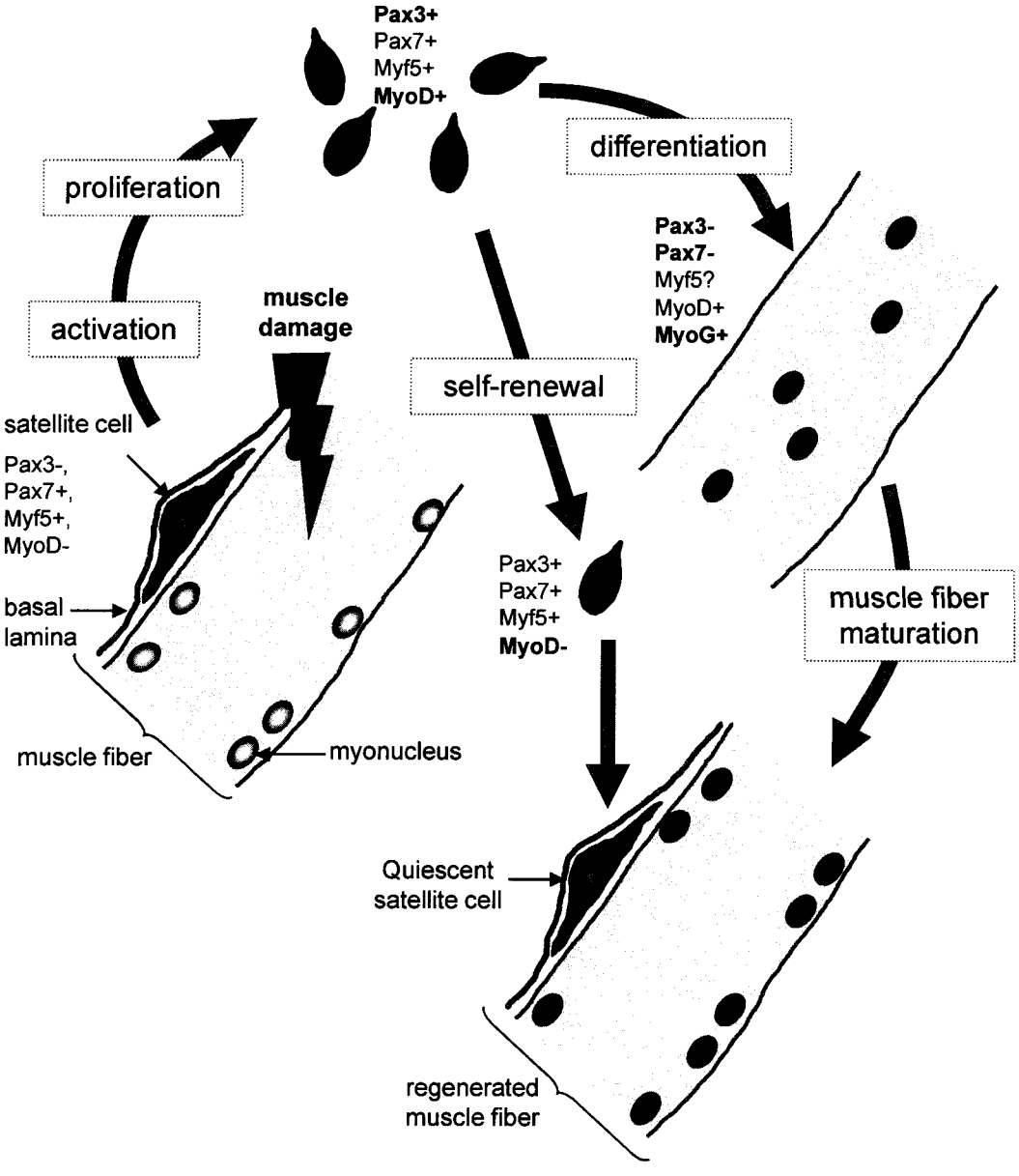


Figure 11. **'Renewal' or 'Differentiation' of satellite cell**

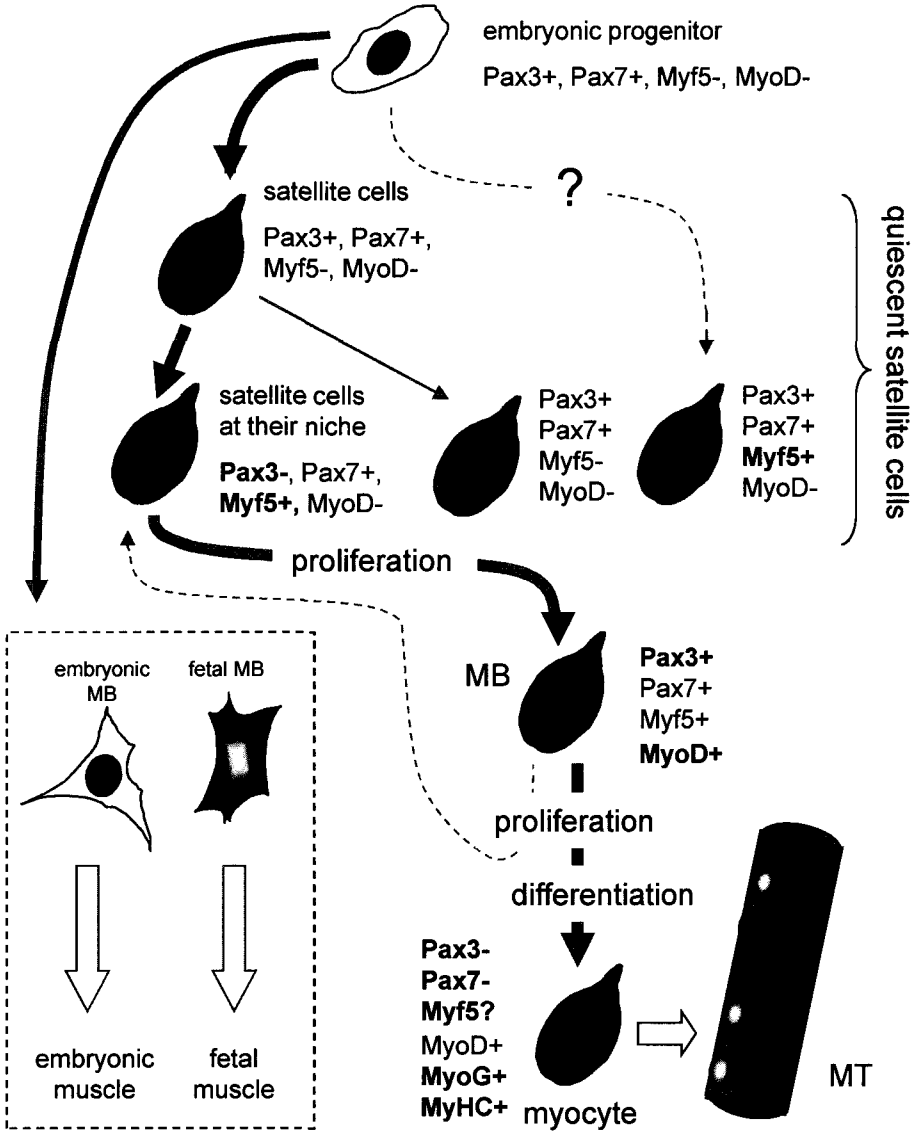


Figure 12. **Molecular markers during 'renewal' or 'differentiation' of satellite cells**

committed satellite cells (Pax7+, Myf5+) further up-regulate MyoD and proliferate and form MBs (figure 11 and 12). Some Pax7+/Myf5+/MyoD+ MBs down-regulate MyoD and become Pax7+/Myf5+/MyoD- quiescent satellite cells (self-renewal), and the other committed satellite cells down-regulate Pax7 and induce the *myog* gene and become myocyte (Pax7-, MyoG+) (Halevy et al., 2004; Olguin and Olwin, 2004; Zammit et al., 2004b) (figure 12). Therefore, Pax7 and MRF may cross-inhibit each other's expression. In culture, Pax7 is able to inhibit the *myog* gene induction by inhibiting MyoD's transactivation properties, and MyoG down-regulates *pax7* expression (Olguin et al., 2007).

2.2. Adult myogenesis; Satellite cell activation; Role of the Notch signalling

For life-time long efficient and sustained skeletal muscle repair, satellite cells first expand by proliferation and then withdrawn from the cell cycle and differentiate to regenerate myofibers (differentiation), and also adult stem cell population need to be maintained by self-renewal.

As seen in fetal myogenesis, the Notch signalling plays a pivotal role in satellite cell activation (Conboy and Rando, 2002; Sun et al., 2007). In agreement with these observations, in parallel to decreased skeletal muscle regeneration capacity with age, the Notch expression level in the satellite cells is decreased (Conboy et al., 2003). The 'canonical' Notch signalling pathway starts with activation of Notch receptors at plasma membrane by ligands of the Delta, Serrate/Jagged, and Lin-12 and Glp phenotype-2 (DSL) family (figure 13B). Both Notch receptors and the DSL ligands are type I single-pass integral membrane proteins with tandem EGF-like

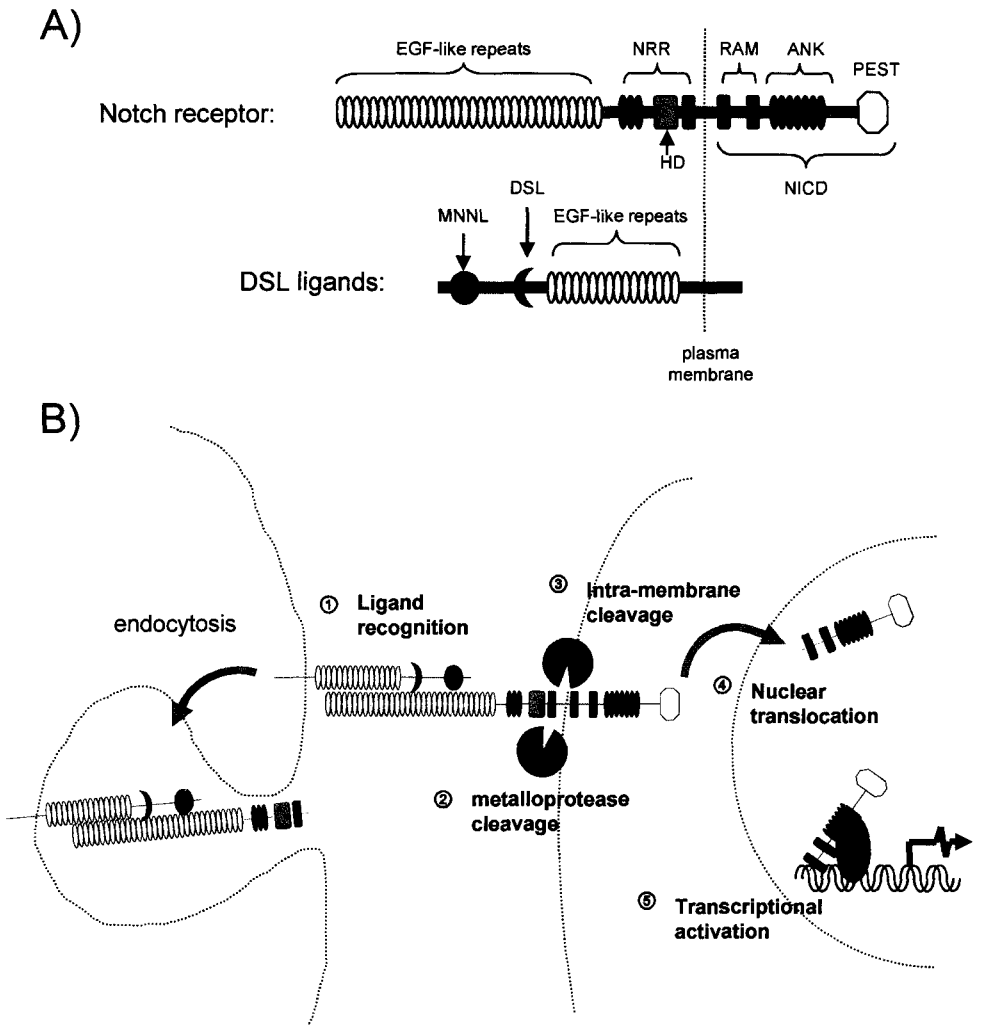


Figure 13. **'Canonical' Notch signalling pathway** A) schematic representation of a prototypical Notch receptor and DSL ligand B) Notch signalling pathway.

NRR; negative regulatory region, (**LNR**; cysteine-rich Lin12/Notch repeat, **HD**; heterodimerization domain), **RAM**; recombination binding protein-Jκ-associate module domain, **ANK**; ankyrin repeat domain, **PEST**; proline-, glutamic acid-, serine- and threonine-rich domain, **MNNL**; module at the N-terminal of Notch ligand domain, **DSL**; Delta/Serrate/Lga-2 family domain; **NICD**; Notch intracellular domain or intracellular Notch (ICN), **RBPJ** or C-promoter-binding factor (CSL).

repeats in their extracellular domains (figure 13A). This ligand/receptor complex formation causes proteolytic release of the Notch intracellular domain (NICD), which tanslocates to the nucleus and regulates target genes as a transcription factor associate with other transcription factors (figure 13B). Therefore, there is no amplification of the signal at the receptor level and irreversible activation in the Notch 'canonical' signalling pathway. The Notch signalling pathway is evolutionarily highly conserved (*C.elegance* to mammals) and required for cell fate determination and tissue/organ development (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999; Chan and Jan, 1998; Fortini, 2009; Kopan and Ilagan, 2009; Lai, 2004).

Some mechanisms by which the Notch signalling pathway maintains activated satellite cells in an undifferentiated state have been proposed. The NICD interacts with MyoD and Myf5 in the nucleus as an active repressor (Kopan et al., 1994). In the limb development in the chick embryo, Delta-1 and Serrate-2 activate the Notch pathway and inhibit muscle differentiation through Pax3, Myf5, and MyoD (Delfini et al., 2000) (figure 14A). Stra13, a basic helix-loop-helix transcriptional repressor, is required for skeletal muscle regeneration. Notch signalling is enhanced in *stra13*-/- MBs, and they exhibit an enhancement in proliferation and defects in muscle differentiation (Sun et al., 2007). An another group also reported that induction of *myog* and other MyoD target genes is repressed by the binding of Stra13 on the E-box in the regulatory region of these target genes (Hsiao et al., 2009). In addition, genetic ablation of the *mastermind-like-1 (maml1)* gene, which encodes an essential co-activator for the NICD, suffers severe muscle dystrophy, and ectopic expression

of MyoD could not convert the *maml1*-/- fibroblasts into myocytes. However, this is not due to a non-functional Notch 'canonical' pathway, MAML1 is also a co-activator of MEF2C. Therefore, the NICD competes with MEF2C for MAML1 upon activation of the Notch signalling and interferes with MEF2C's pro-myogenic activity (Shen et al., 2006) (figure 14B). In addition, a recent study documented that Notch signalling inactivates p38MAPK, which enhance muscle differentiation by phosphorylation of several myogenic regulatory factors including MEF2s (Cox et al., 2003), by up-regulation of Mitogen activated protein (MAP) Kinase phosphatase-1/ Dual specificity phosphatase-1 (MKP1/Dusp1) (Kondoh et al., 2007). Megf10, which is a multiple epidermal growth factor repeat transmembrane protein, is a quiescent satellite cell marker gene, which is expressed in the satellite cells and MBs but not in the myofibers. Megf10 is required for self-renewal of the satellite cells. Reduction of Megf10 causes *myog* activation and precocious differentiation by impinging Notch signalling. Therefore, Megf10 regulates balance between self-renewal and differentiation of the satellite cells through the Notch signalling (Holterman et al., 2007) (figure 13).

A recent study also indicates that the Wnt signalling interferes with the Notch signalling to shift the cells from proliferation to differentiation. Glycogen synthase kinase-3β (GSK3β) is a mediator of this crosstalk. GSK3β is active under the influence of the Notch signalling, but it is inactivated by Wnt signalling (Brack et al., 2008). Upon muscle injury, myofibers up-regulate Wnt ligands transcriptionally, and secreted the Wnt ligands from injured myofibers. The Wnt ligands act on the satellite cells, and activate β-catenin, which is a co-activator of

the down-stream transcription factor of the 'canonical' Wnt signalling (Otto et al., 2008). In particular, Wnt7a stimulates symmetric proliferation of satellite stem cells without affecting on the differentiation of the MBs. Therefore, Wnt7a plays role for expansion of the satellite stem cell population by self-renewing (Le Grand et al., 2009) (figure 13).

2.3. Adult myogenesis; Satellite cell differentiation

Once the satellite population is activated by exercise or muscle injury, the fate of the activated satellite cells will be determined. However, to achieve both efficient regeneration of damaged skeletal muscle and maintenance of 'stem' cell pool for life-time long, satellite cells have to commit to both of these very different fates. A few models have been proposed to explain this characteristic of the satellite cell, such as asymmetric cell division (Conboy and Rando, 2002; Conboy et al., 2007; Shinin et al., 2006) (figure 14, 15, and 16), symmetric cell division followed by a stochastic cell fate determination (Halevy et al., 2004; Olguin and Olwin, 2004; Zammit et al., 2004b), and the niche models (Day et al., 2007; Holterman et al., 2007; Kuang et al., 2008; Kuang et al., 2007) (figure 17). They are not necessary mutually exclusive to each other.

2.3.1. Asymmetrical cell division by Notch signalling pathway

For activation of skeletal muscle satellite cells, Notch 'canonical' pathway plays pivotal role, and several Notch signalling regulators in the satellite cells has been identified and characterized.

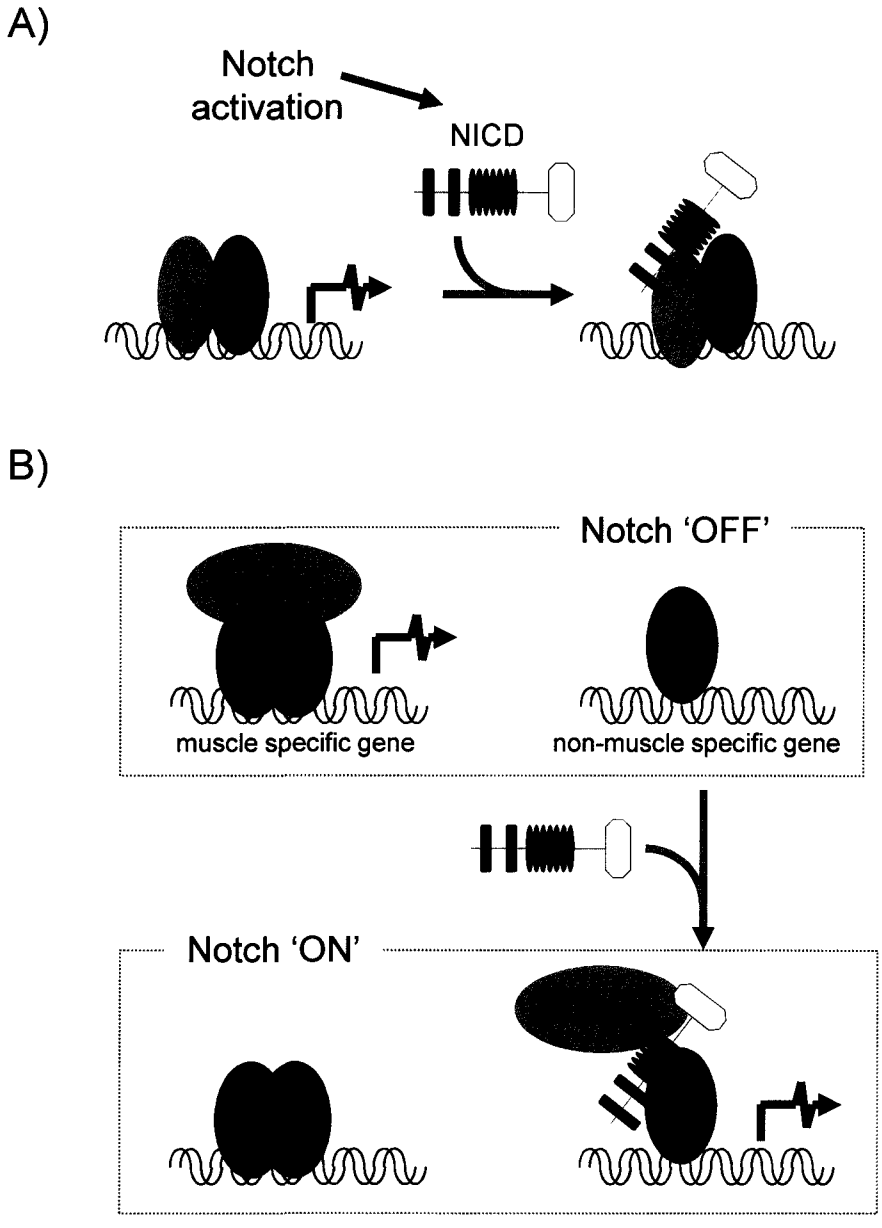


Figure 14. **Model of inhibition of myogenesis by Notch signalling**

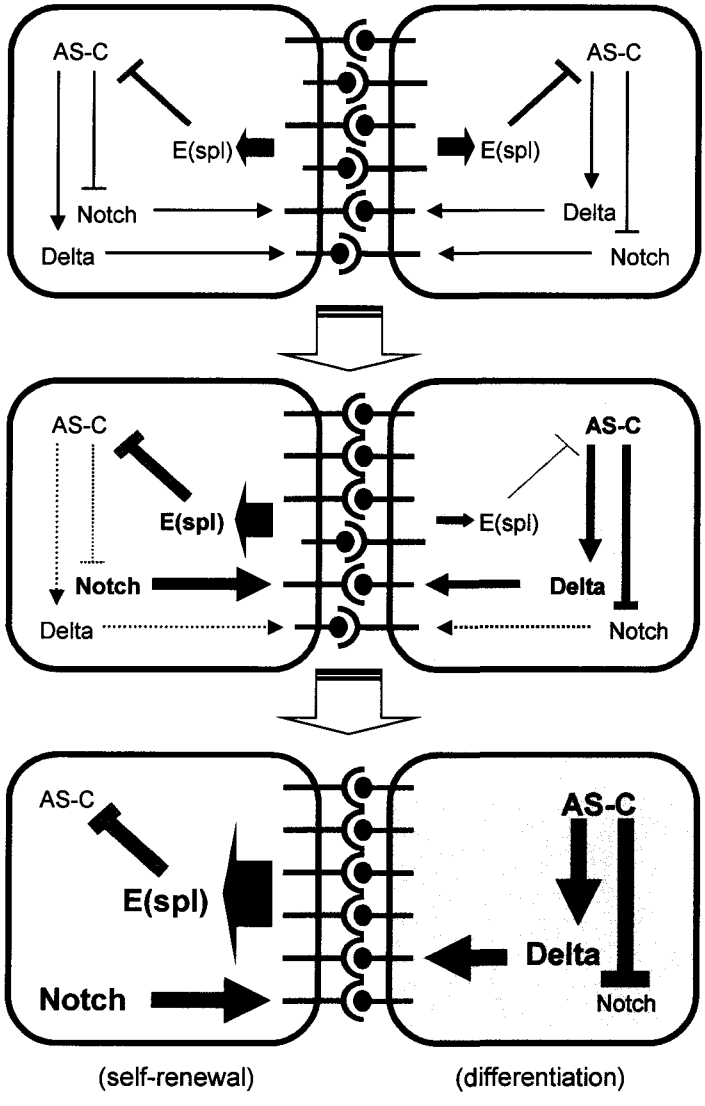


Figure 15. **Model of Lateral inhibition**

AS-C; achaete-scute complex, **E(spl)**; HES family of bHLH regulators

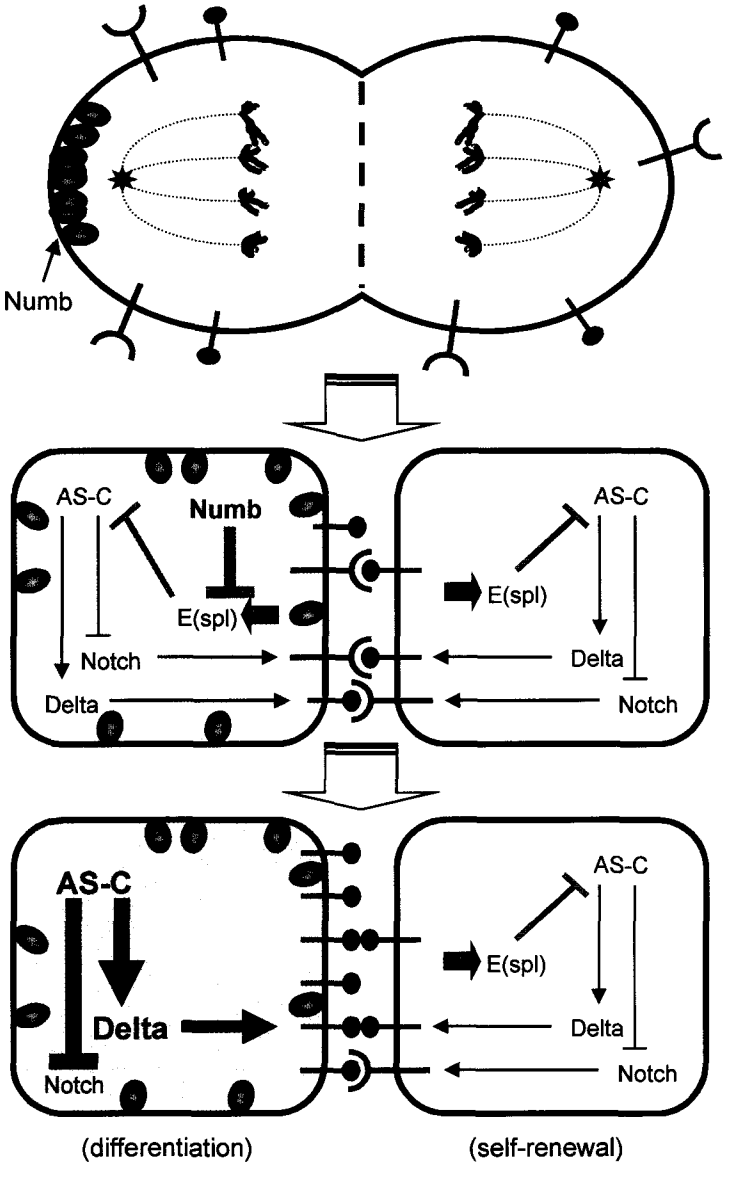


Figure 16. **Asymmetrical cell division with Numb**

AS-C; achaete-scute complex, **E(spl)**; HES family of bHLH regulators

2.3.1.1. Lateral inhibition

Signalling molecules in the Notch signalling pathway are regulated by feedback loops. Since a Notch target gene, the E(spl)/HES family of bHLH regulators, antagonises the activity of the *achaete-scute complex (as-c)* gene, which up-regulates Notch and down-regulates Delta, the Notch activation enhances Notch and represses Delta expression (figure 15). These positive (for Notch expression) and negative (for Delta expression) feedback loops causes initially equivalent cells to be two different characteristic cells (signal sending (Delta expressing cell) and signal accepting (Notch expressing cells). By this 'lateral inhibition', the Notch signalling specifies different cell fates in adjacent cells (figure 15). This 'lateral inhibition' plays key role in many developmental scenarios in coordination with other positional and temporal cues in order to specify different cell types (Fortini, 2009; Kopan and Ilagan, 2009).

2.3.1.2. Role of Numb protein in asymmetrical cell division

Activation of Notch1 signalling promotes proliferation of satellite cells in the Pax3+ ('pre-MB') state. Activated satellite cells divide asymmetrically due to uneven distribution of Numb, which is an antagonist of Notch1 signalling (figure 16). Increased Numb expression shifts the satellite cells in 'pre-MB' state to MB fate (Myf5+, MyoD+, Desmin+, and Pax7+). During mitosis, after activation of the satellite cells, Numb localized one of the cortical crescents; as a result, progenys have different amount of Numb proteins. Therefore, the daughter cells of the

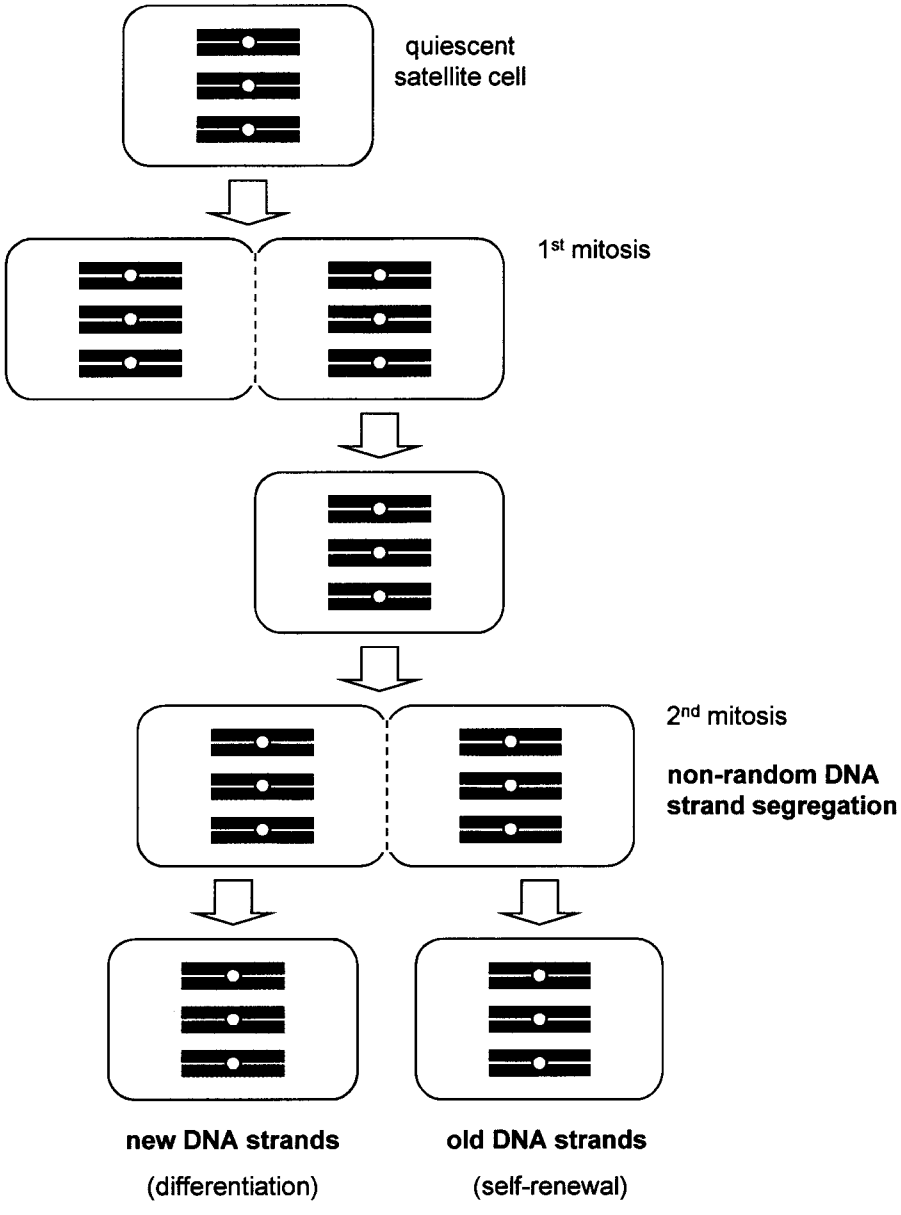


Figure 17. **Non-random DNA segregation**

activated satellite cells become heterogeneous in myogenic commitment (figure 16). Thus, the balance between Notch1 and Numb generates muscle precursors for self-renewal of the satellite cells and MB for growth and regeneration of skeletal muscle by asymmetric cell division (Carmena et al., 1998; Conboy and Rando, 2002; Lu et al., 1998; Shinin et al., 2006) (figure 16). With myofibers, Numb proteins localize the basal lamina side in the dividing muscle progenitors (Holowacz et al., 2006). Therefore, positional information regulates asymmetric cell division; self-renewal and differentiation (Shinin et al., 2006).

2.3.1.3. Asymmetrical cell division by non-random DNA strand segregation

Not only Numb proteins but also DNA strands segregate asymmetrically during the mitosis of the myogenic precursors (figure 17). After the first cell division, during the second mitosis, all old DNA strands tend to segregate together into one of the daughter cells. The old DNA strand receiving cells are self-renewing cells, the new DNA strands co-segregate into the differentiating cells assessed by a stem cell marker Sca1 and the differentiation marker Desmin (Conboy et al., 2007) (figure 17). Although this asymmetric cell division occurs a small portion of the satellite cells, similar co-segregation of the DNA strands observed in epithelial and neural stem cell but not in hematopoietic stem cell (Conboy et al., 2007; Karpowicz et al., 2005; Kiel et al., 2007; Shinin et al., 2006; Shinin et al., 2009). This asymmetric cell division capacity of the muscle stem cell is lost quickly in cell-culture setting (Conboy et al., 2007; Shinin et al., 2006) suggesting that it is context dependent and positional information is required (Kuang et al., 2008). However, Numb proteins

were observed to be co-segregated with the old DNA strands instead, and the reason for this discrepancy is not clear (Shinin et al., 2006).

2.3.2. Symmetric cell division followed by a stochastic cell fate determination

Upon stimulation, activated quiescent satellite cells (Pax7+, MyoD-) up-regulate MyoD and symmetrically proliferate and become MB (Pax7+, MyoD+). Some of these MBs stochastically down-regulate MyoD and become quiescent satellite cells (Pax7+, MyoD-) for self-renewal, the others up-regulate MyoG and down-regulate Pax7 to enter differentiation program (Pax7-, MyoD+, MyoG+) (Halevy et al., 2004; Olguin and Olwin, 2004; Zammit et al., 2004b).

2.3.3. Asymmetrical cell division; positional effect (niche regulation)

Three important components of the satellite cell niche are the host myofibers, the basal lamina, and the micro-vasculature. The host myofibers communicate with the satellite cells and regulate satellite cell functions (Charge and Rudnicki, 2004; Molgo et al., 2004; Tatsumi et al., 2006). The basal lamina is an extracellular matrix (ECM), which contains laminin, collagens and proteoglycans. The satellite cells attach to the basal lamina, which is essential for the stem cell identity (Blanpain et al., 2004; Fuchs et al., 2004). In addition, a variety of extrinsic signals from fibroblasts, muscle-resident stem cells, macrophages, and circulatory system transduce through the basal lamina and capillaries/vascular endothelial cells (Christov et al., 2007). In addition, the satellite cells express integrin $\alpha 7\beta 1$ receptors, and they are localized at the basal side of the satellite cells and interact with the laminin on the basal lamina (Burkin and Kaufman, 1999). At the apical

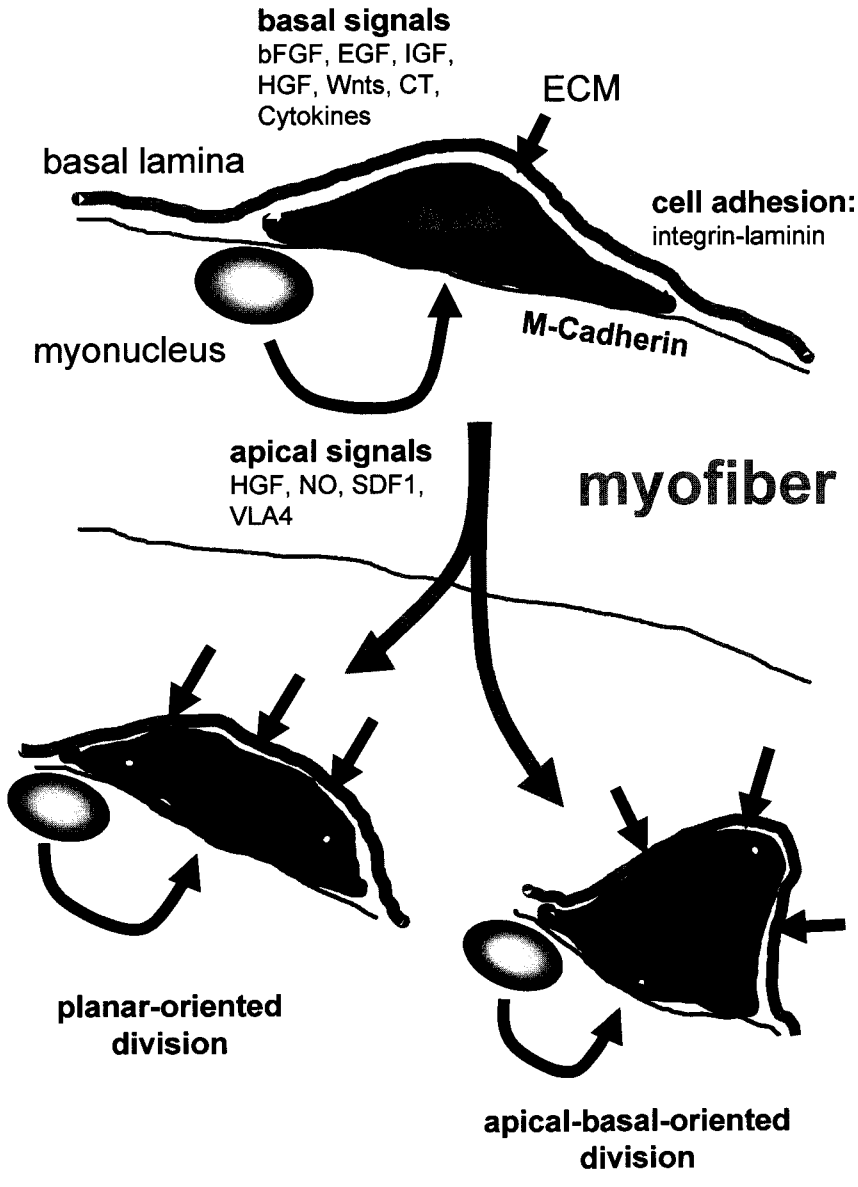


Figure 18. **Asymmetrical cell division; Niche effect**

side, the satellite cells communicate with the host myofibers through M-Cadherin (Cornelison and Wold, 1997). Therefore, the satellite cells receive different signals from apical and basal side (figure 18).

In this niche, two daughter cells produced by a planar-oriented cell division from an activated satellite cell are exposed to the identical signals. However, an apical-basal-oriented cell division generates two daughter cells exposed to the different signals (figure 18). As a result, these two daughter cells are specified symmetrically in former case and asymmetrically in the later case. In fact, the daughter cell attaches with the basal lamina adapts a self-renewal fate, and the other loses to contact with the basal lamina adapts a differentiation fate (Kuang et al., 2007). There are still some discrepancies, but asymmetric localization of Numb protein and asymmetric segregation of DNA strands during mitotic cell division in the activated satellite cells might require this particular niche (Conboy et al., 2007; Kuang et al., 2007; Shinin et al., 2006).

2.3.4. Cytokines and Growth factors that satellite cells are exposed to

Upon skeletal muscle injury, satellite cells are activated for muscle repair. The damaged myofibers and the activated satellite cells secrete chemo-attractants for monocytes and macrophages infiltration, which causes inflammation reactions. Interestingly, the blockade of inflammatory cell infiltration impairs muscle repair. Macrophage inflammatory protein-1β (MIP1β) or vascular endothelial growth factor (VEGF) stimulate the satellite cell proliferation and differentiation (Lescaudron et al., 1999), and the activated satellite cells secrete VEGF and attract

monocytes and macrophages, which support survival of the activated satellite cells (Chazaud et al., 2003). A variety of inflammatory cytokines affect on the myogenesis, such as Interleukin-4 (IL-4) (MB fusion, from MT) (Horsley et al., 2003), Leukemia Inhibitory Factor (LIF) (maintenance of the 'stem' state) (Murray and Edgar, 2001), Transforming Growth Factor β (TGFβ) (fibrotic disorder, from macrophage) (Vidal et al., 2008), IL-6 (satellite cell proliferation, from myofibers) (Serrano et al., 2008), and Tumour Necrosis Factor α (TNFα) (early phase of differentiation of MB, from myofibers) (Li and Schwartz, 2001). In addition to the initiation of inflammatory response, Matrix metalloproteinase (MMP) activity mediated release of growth factors, which is being bound to the ECM proteins such as proteoheparan sulfates, from myofibers involves in muscle damage repair (Carmeli et al., 2004; Husmann et al., 1996). In addition, Fibroblast Growth Factors (FGFs) (Clegg et al., 1987; DiMario et al., 1989), Hepatocyte Growth Factor (HGF) (Tatsumi et al., 1998), Insulin-like Growth Factor 1 (IGF1) (Machida and Booth, 2004; Perrone et al., 1995), Brain-Derived Neurotrophic Factor (BDNF) (Mousavi and Jasmin, 2006), Calcitonin (CT) (Fukada et al., 2007), Stromal-Derived Factor 1/Chemokine (C-X-C motif) Ligand-12 (SDF1/Cxcl12) (Ratajczak et al., 2003; Sherwood et al., 2004), epidermal growth factor (EGF) (Golding et al., 2007), Tumour necrosis factor-like WEAK inducer of apoptosis/Tumour Necrosis Factor (ligand) Superfamily member-12 (TWEAK/TNFSF12) (Dogra et al., 2006; Girgenrath et al., 2006), Nitric Oxide (NO) (Tatsumi et al., 2006; Wozniak and Anderson, 2007), Integrin β1 (VLA4) (Rosen et al., 1992), Laminin (Burkin and Kaufman, 1999), M-Cadherin (CDH15) (Irintchev et al., 1994), and

Myostatin/Growth Differentiation Factor-8 (GDF8) (McCroskery et al., 2003) have been shown to regulate myogenesis. As discussed above, Wnt (Brack et al., 2007; Polesskaya et al., 2003) and Delta1 (Conboy et al., 2003; Kuang et al., 2007; Schuster-Gossler et al., 2007) also regulate adult myogenesis (summarized in Table 1).

Cardiotrophin-1 (CT-1) signalling pathway

As discussed above, despite the fact that a variety of cytokines and growth factors affect on myogenesis at multiple stages, a detailed understanding of their corresponding signal transduction pathways and their down-stream targets are still not clear. Is there any common target?

Cardiotrophin-1 (CT-1) is a member of the IL-6 family, which is comprised of IL-6, IL-11, Leukemia inhibitory factor (LIF), Ciliary Neurotrophic Factor (CNTF), Cardiotrophin-Like Cytokine (CLC), and Oncostatin M (OSM). Their down-stream target genes are regulators of differentiation, proliferation, survival, apoptosis, and especially haematopoiesis. In addition, pro- and anti-inflammatory response and acute phase and immune response are controlled by the genes regulated by these cytokines. CT-1 was originally identified in conditioned medium from embryoid bodies (Pennica et al., 1995). In developing embryos, CT-1 is expressed in heart, skeletal muscle, liver and dorsal root ganglia (Sheng et al., 1996). In adults, human CT-1 mRNA is detected in the heart, skeletal muscle, ovary, colon, prostate and testis, and in fetal kidney and lung (Pennica et al., 1996b). The functions of CT-1 in the cardiovascular system have been characterized. The amount of CT-1 circulating in the patients suffering from ischemic and valvular heart disease is increased (Freed et al., 2003). CT-1 protects cardiomyocytes from apoptosis (Brar et al., 2001; Sheng et al., 1996) and is involved in regeneration of cardiac muscle after infarction (Freed et al., 2005). Exogenous CT-1 causes cardiac hypertrophy *in vitro*

(Sheng et al., 1996). CT-1 also supports survival of sciatic motoneurons (Pennica et al., 1996a). However, down-stream molecular events are still not well characterized, and the role of CT-1 in embryonic myogenesis and adult muscle growth and regeneration has not, thus far, been characterized.

1.1. Signalling pathway of IL-6 family; Ligand-Receptor complex formation

IL-6 family cytokines are structurally related. They have four α-helices, which are organized in an up-up-down-down orientation (figure 19A). IL-6 and IL-11 have all straight helices, but LIF, OSM, and CNTF have a kinked helix (Bravo and Heath, 2000; Deller et al., 2000) (figure 19A). It suggested that these structural differences cause the recruitment of a different pair of the signalling receptors. IL-6 and IL-11 form a complex with gp130/gp130 homodimer, but the others complex with gp130 heterodimer. In addition to the signalling receptor dimers, IL-6, IL-11, CNTF, and CLC also bind a non-signalling α receptor such as IL-6Rα, IL-11Rα, and CNTFRα (for both CNTF and CLC) (Heinrich et al., 2003; Heinrich et al., 1998; Senaldi et al., 1999). The α receptor for CT-1 was predicted, but it has not yet been characterised (Robledo et al., 1997). The signalling complex formation of IL-6 cytokine family is summarized in figure 19B.

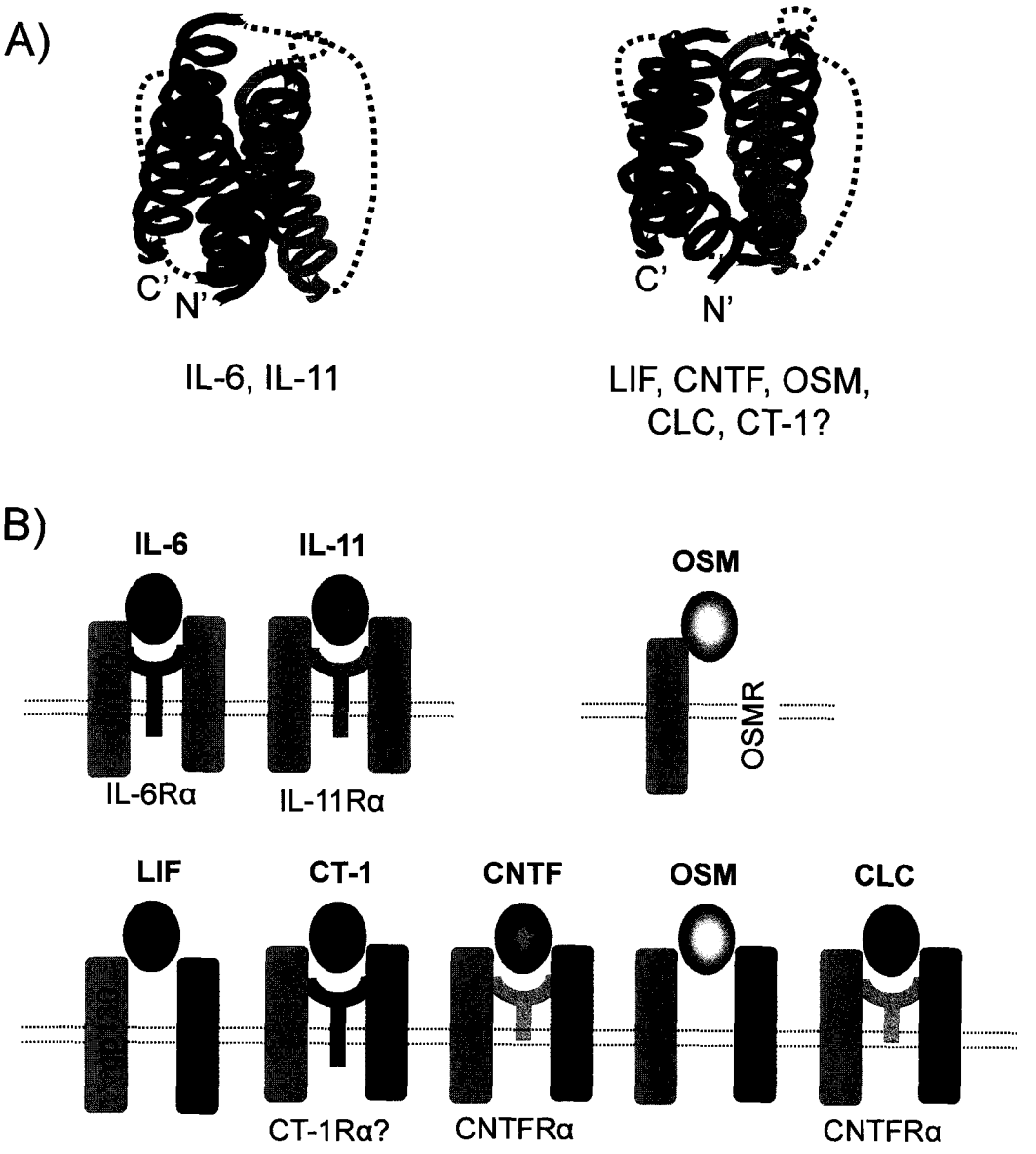


Figure 19. **IL-6 family cytokines**

A) Structure model of IL-6 family cytokines. B) Ligand/receptor combinations.

2.1. Signalling pathway of IL-6 family; Janus kinase (JAK) activation

Upon formation of the requisite complex with the respective cytokine, the oligomeric receptor complex transduces the signal to the nucleus through the Janus Kinase (JAK) to Signal Transducer and Activator of Transcription (STAT) for gene regulation (Heinrich et al., 2003) (figure 20). There are three kinases such as JAK1, JAK2, and Tyrosine Kinase-2 (TYK2) that are responsible for transducing signal from the receptor complex, and they constitutively associate with glycoprotein 130 (Gp130) and LIF β-receptor (LIFRβ) receptors (Hermanns et al., 1999; Lutticken et al., 1994; Radtke et al., 2005; Stahl et al., 1994). Since loss of functional JAK1 causes impaired IL-6 signalling, JAK1 is essential for the gp130 mediated signal transduction (Guschin et al., 1995; Rodig et al., 1998). A major interaction region of gp130/ LIFRβ/OSMR receptors with JAKs is the conserved membrane proximal region, the proline-rich box1 (Haan et al., 2000; Murakami et al., 1991; Radtke et al., 2002). The FERM (Four-point-one, Ezrin, Radixin, and Moesin) domain in the N-terminal region of JAKs is required for interaction with gp130/LIFRβ/OSMR (Haan et al., 2001) (figure 20). A mutation of the 652 tryptophan (W652) within the box1 of the Gp130 to alanine (W652A) eliminates the cytokine dependent activation of JAK1 without affecting on the JAK1 association with the receptor. This mutation causes dominant negative inhibition for the JAK1 activation suggesting that cytokine dependent activation of JAK1 requires dimer formation

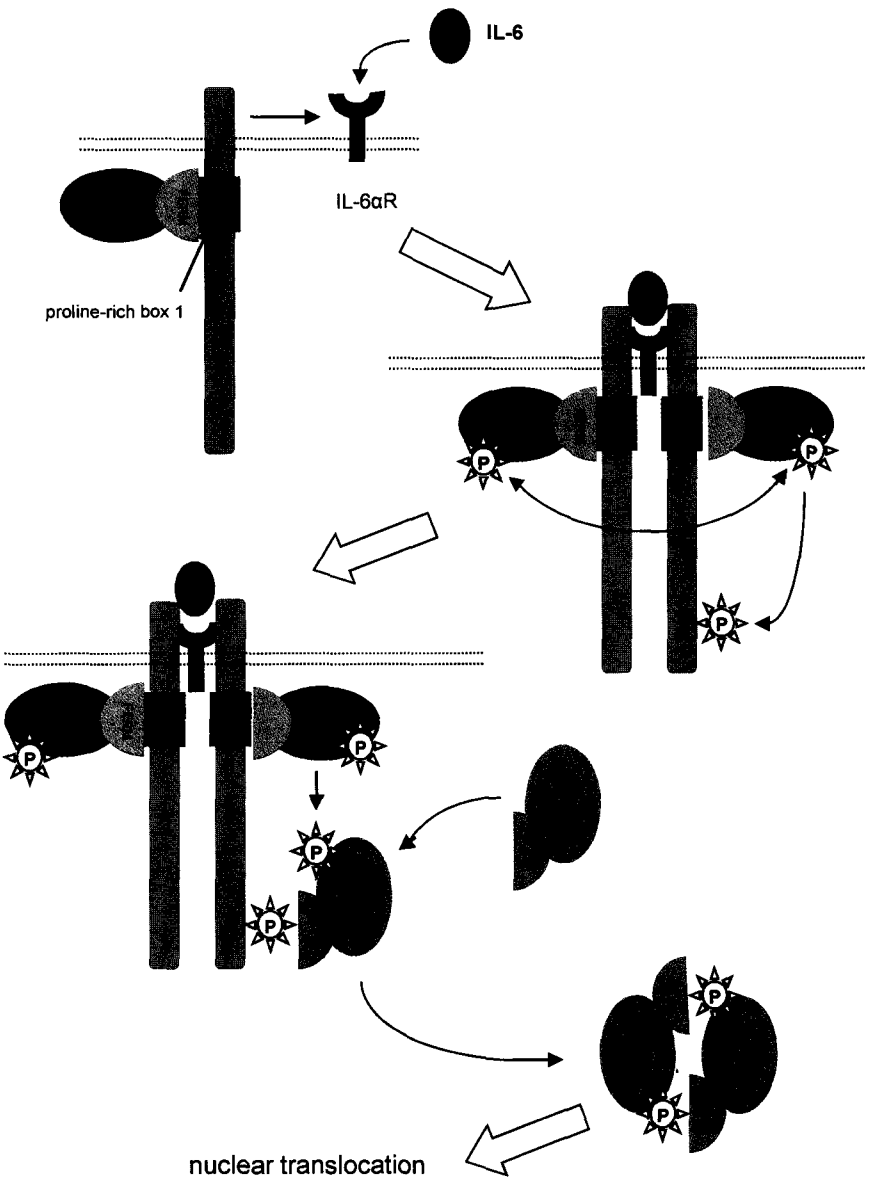


Figure 20. **IL-6 family cytokines activate JAK/STAT signalling pathway**

of the receptor, which is not required for JAK1 association with gp130 (Haan et al., 2002).

2.2. Signalling pathway of IL-6 family; STAT activation

Down-stream targets of JAKs are STAT transcription factors. STATs are sequence specific DNA binding (consensus sequence; $TT(N)_{4-6}AA$ (Seidel et al., 1995)) transcription factors, and STAT family consists of seven different genes, such as STAT1, -2, -3, -4, -5a, -5b, and -6, and a few isoforms are generated by the alternative splicing of the mRNAs (Becker et al., 1998; Chen et al., 1998; Vinkemeier et al., 1998).

STAT3 and STAT1 are activated by Gp130, and LIFRβ/OSMR activate STAT3, STAT1, and STAT5 (Heinrich et al., 1998; Lai et al., 1995). These cytokine receptors have several STAT binding motif (YXXQ) in their cytoplasmic tails, and the STAT proteins are recruited to the activated receptor through the interaction of this YXXQ motif (Y (Tyr) is phosphorylated by activated JAKs) and the Src homology domain-2 (SH2) of STAT proteins (Gerhartz et al., 1996; Heim et al., 1995; Hemmann et al., 1996; Stahl et al., 1995). STAT proteins are phosphorylated by JAKs at the single tyrosine (705 for STAT3, 701 for STAT1), and phosphorylated STATs dissociate from the receptor and form dimer through the phosphor-tyrosine/SH2 inter-molecular interaction (Kaptein et al., 1996; Shuai et al., 1994; Shuai et al., 1993a; Shuai et al., 1993b). The STAT dimers translocate to nucleus and activate target genes (Zhang et al., 2000b), and this tyrosine phosphorylation is essential for STAT nuclear translocation (Gough et al., 2009)

(figure 20). However, some tyrosine phosphorylated STAT1 and -3 also localizes in the cytoplasm and membrane rafts with caveolin-1 and heat-shock protein-90 (Sehgal et al., 2002; Shah et al., 2002).

STAT1, -3, -5a, and -5b are also serine-phosphorylated upon cytokine stimulation. The phosphorylation at the 727 Serine (S727) of STAT3 may further enhance transcriptional activity of STAT3, but some reported that this phosphorylation reduces the STAT3 activity (Abe et al., 2001; Beuvink et al., 2000; Haq et al., 2002; Lim and Cao, 2001; Nair et al., 2002; Sanceau et al., 2000; Schuringa et al., 2000; Su et al., 1999). A few studies show that the phosphorylation of S727 is MAKP independent (Abe et al., 2001; Chung et al., 1997), and the others document that it depends on PKCδ (Jain et al., 1999; Schuringa et al., 2001). However, the role of this serine phosphorylation of STAT may depend on cellular context (Aziz et al., 2007; Gartsbein et al., 2006; Park et al., 2008; Wang et al., 2005b); therefore, further characterization is required.

3.1. Signalling pathway of IL-6 family; activation of MEK/ERK kinases

Dimerization of the cytokine β-receptor (gp130, LIFRβ, and OSMR) activates not only the JAK/STAT signalling pathway but also the MAPK cascade. SH2 domain containing tyrosine phosphatase (SHP2) plays a critical role for MAPK activation by Gp130 and LIFRβ (figure 20). Phosphorylation at Y759 in Gp130 and Y974 in the LIFRβ are required for the SHP2 binding (Schiemann et al., 1997; Stahl et al., 1995). OSMR activates the same kinase cascade recruiting SH2 and collagen homology domain containing protein (Shc) at phosphorylated Y861 instead

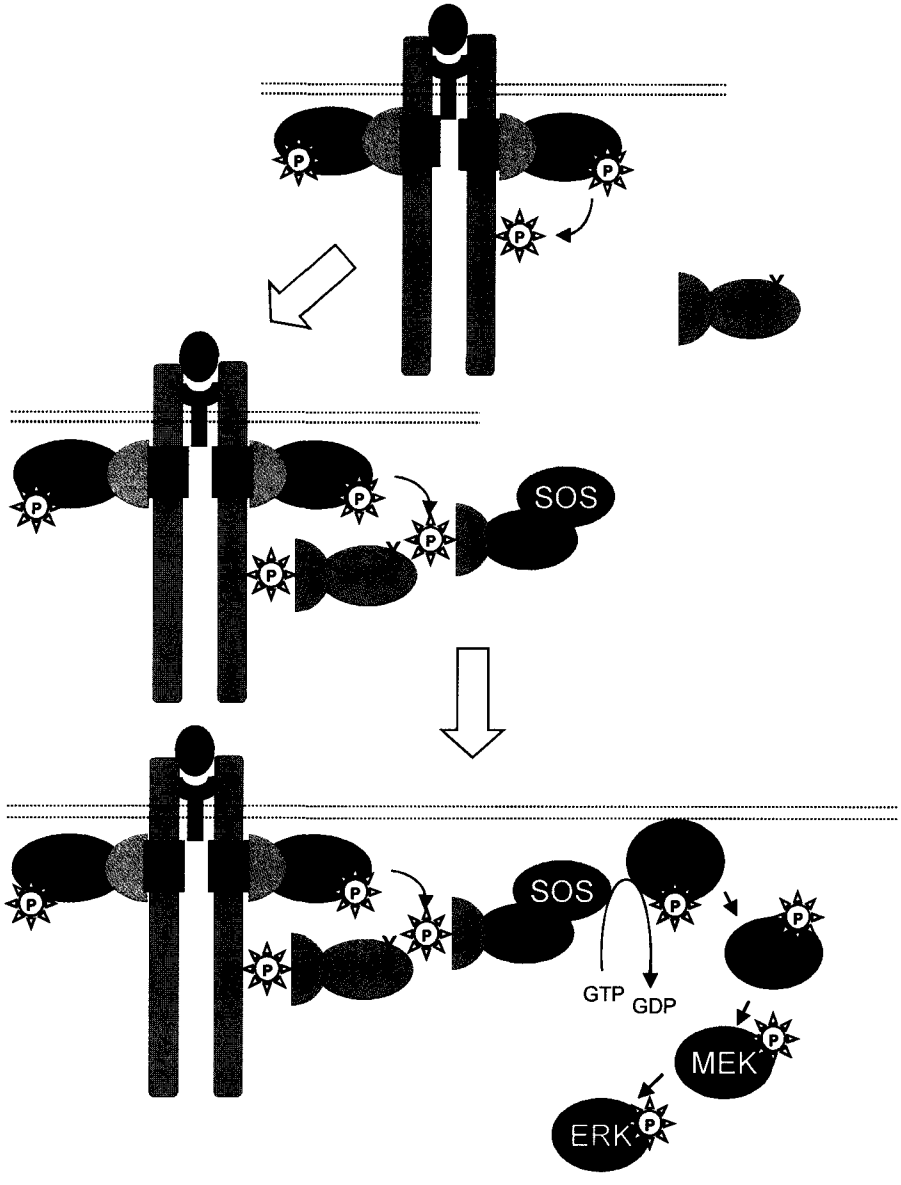


Figure 21. **IL-6 family cytokines activate Ras/Raf/MEK/ERK cascade**

(Hermanns et al., 2000). Phosphorylation of the tyrosine residue in SHP2 requires JAK1 but not JAK2 or TYK2 (Schaper et al., 1998). It is speculated that the phosphorylated tyrosine amino acid provides the binding sites for SH2 domain of Growth factor receptor bound protein (Grb2)/Son of Sevenless (SOS) and/or Grb2 associated binder 1 (Gab1) complex. This complex formation leads to activation of membrane anchored Ras, and it initiates MAPK cascade (Ras-Raf-MEK-ERK) (Fukada et al., 1996; Gu et al., 1998; Hermanns et al., 2000; Holgado-Madruga et al., 1996; Schaper et al., 1998; Schiemann et al., 1997) (figure 21). Since Gab1 is a scaffolding adaptor protein with pleckstrin homology (PH) domain, it can interact with the Grb2, SHP2, phosphatidylinositol 3-kinase (PI3K), Crk (v-crk sarcoma virus CT10 oncogene homolog), Phospholipase Cγ, and c-Met (hepatocyte growth factor receptor) (Holgado-Madruga et al., 1996; Liu and Rohrschneider, 2002; Schaeper et al., 2000). IL-6 family cytokines are known to activate PI3K/Akt (PKB), and p38MAPK, JNK as well (Bode et al., 2001; Hideshima et al., 2001; Negoro et al., 2001; Schuringa et al., 2001; Zauberman et al., 1999).

4.1. Role of CT-1signalling in myogenesis

CT-1 is expressed in skeletal muscle during embryo development (Pennica et al., 1996a; Pennica et al., 1996b). In regenerating and overloaded skeletal muscles, CT-1 proteins are expressed in satellite cells and infiltrated macrophages. Therefore, it has been suggested that roles of CT-1 in skeletal muscle regeneration and hypertrophy (Nishikawa et al., 2005).

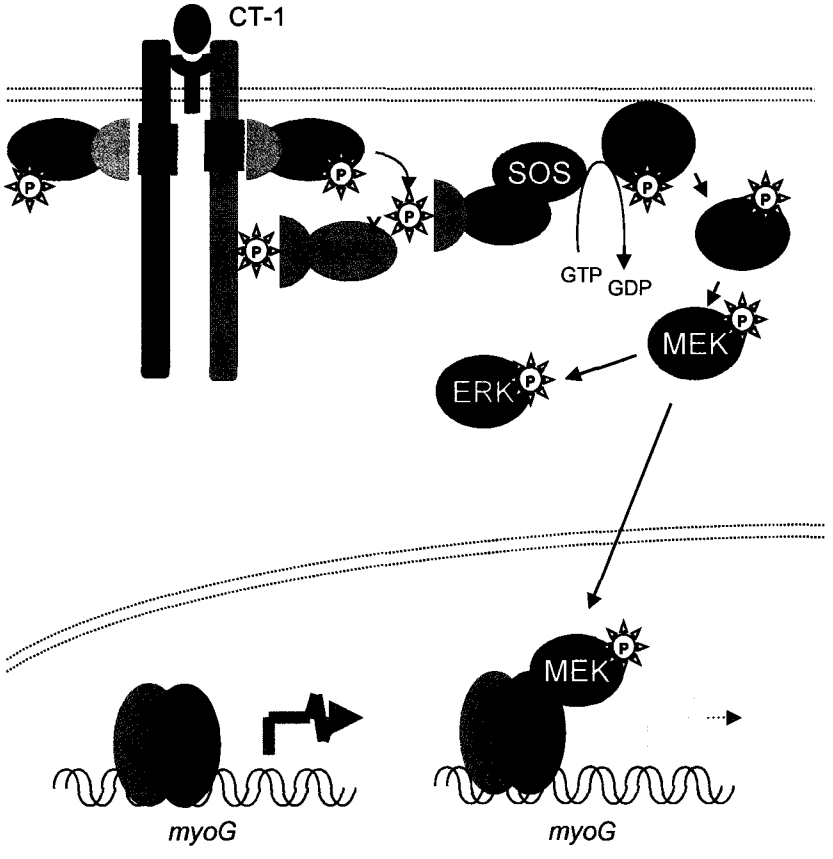


Figure 22. **Inhibitory effect of CT-1 on MyoD through MEK activation**

Gp130 is expressed in skeletal muscle (Arimura et al., 2005). IL-6 is transiently produced from growing myotubes, and IL-6 stimulates satellite cell proliferation in a paracrine manner through STAT3 activation (Serrano et al., 2008). During preparation of our manuscript (Chapter III), Wang et al. (2008b) documented that the JAK1/STAT1/STAT3 pathway is required for MBs proliferation and actively prevent the MBs from precocious differentiation, and the JAK2/STAT2/STAT3 pathway has a pro-myogenic effect (Wang et al., 2008b). LIF inhibits an early phase of myogenesis through activation of ERK but independent of STAT3 activation (Jo et al., 2005). Therefore, originally STAT3's role in myogenesis was proposed as a negative regulator of myogenesis by interfering with MyoD (Kataoka et al., 2003), as we documented (Chapter II), activated STAT3 may not be responsible for the inhibitory effect of CT-1 on myogenesis. CT-1 inhibits skeletal muscle differentiation through activation of MEK, which interacts with MyoD and inhibits MyoD's transcriptional activation properties (figure 22). Therefore, CT-1 does not affect on the muscle specification by MyoD and Myf5 expression in the MBs but prevents the terminal differentiation by interfering with the *myog* gene induction by MyoD. Thus, we propose that CT-1 maintains the undifferentiated state of muscle progenitors through activation of MEK (Miyake et al., 2009).

Tumour Growth Factor β (TGFβ) signalling pathway

TGFβ superfamily consists of TGFβs, BMPs, Growth and differentiation factors (GDFs), Activins and Nodal. They are key regulators for embryonic development and tissue morphogenesis (Feng and Derynck, 2005). TGFβ superfamily proteins believed to be secreted as a disulfide-linked homo- or hetero-dimer. Extracellular matrix (ECM) associated proteins trap secreted TGFβ proteins and sequestrate these growth factors from the receptors. Proteolytic cleavage releases TGFβ ligands from the ECM (Annes et al., 2003).

Processed TGFβ superfamily proteins bind to the cell surface receptors (type II receptors), and then the receptor complex phosphorylates intracellular effecter proteins (receptor Smads; R-Smads), which translocate to the nucleus and regulate target gene expression (Derynck and Zhang, 2003; Shi and Massague, 2003). In addition to this TGFβ 'canonical' signalling pathway, R-Smad independent 'non-canonical' signalling regulates TGFβ response and Smad activity (Derynck and Zhang, 2003).

1.1. 'Canonical' TGFβ signalling pathway; ligand/receptor complex formation

TGFβ ligand/cell surface receptor complex contains 'type I' and 'type II' receptors. Although they are structurally similar transmembrane serine/threonine kinase, the type I receptors have Glysine/Serine-rich 'GS-rich sequence' in their cytoplasmic tail. Without ligand binding, they form homodimers and are inactive kinases. Upon ligand binding, the ligands and the receptors form a stable complex, which consists two of each type I and II receptor and the ligand (figure 23). The type II receptor

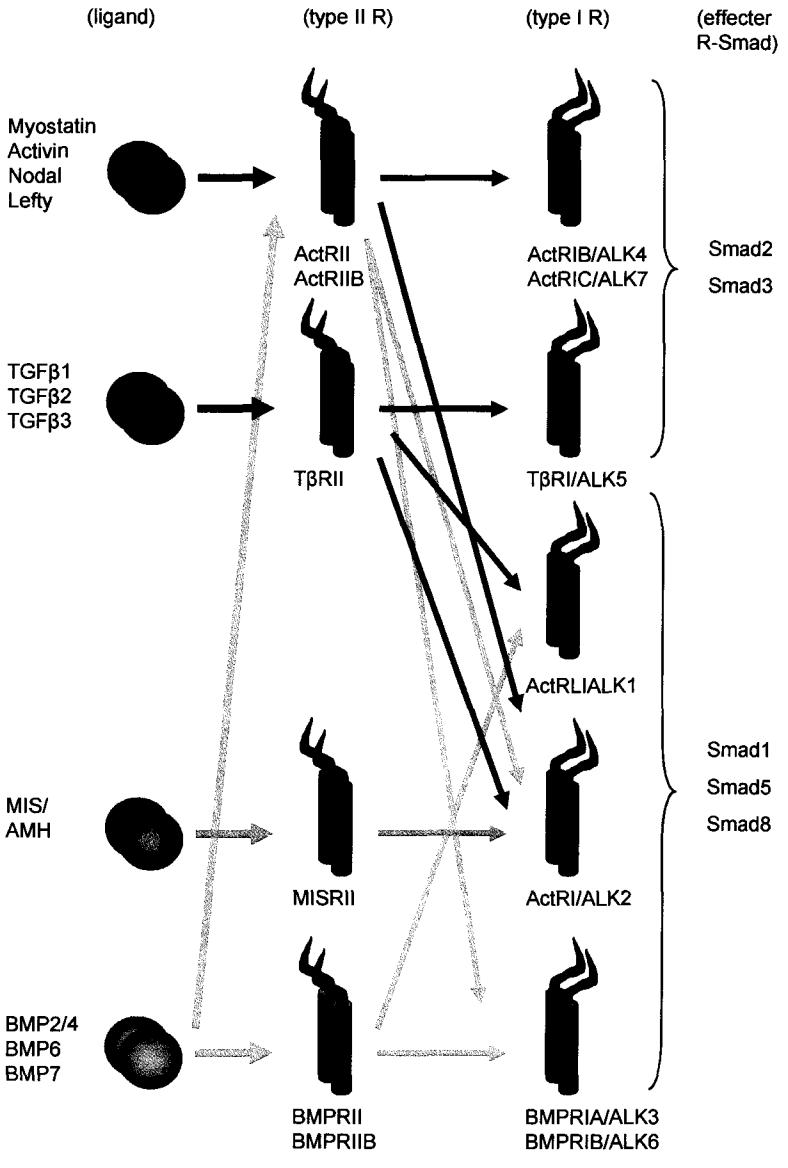


Figure 23. **Ligand/receptor combinations of TGFβ family cytokines**

phosphorylates serine residues in the 'GS-rich sequence' in the type I receptor. This phosphorylation initiates the auto-phosphorylation of the type I receptor, and then the active type I receptor phosphorylates R-Smads associated with the receptor (Derynck and Zhang, 2003; Shi and Massague, 2003). The specific interaction between the type I receptor and the R-Smad is mediated by the L45 loop of the type I receptor and the L3 loop in the MH2 domain of the R-Smad (Lo et al., 1998). Specific combinations of the type I with type II receptors for a given TGFβ superfamily ligand are summarised in figure 23.

Interestingly, although there are at least 29 genes encoding TGFβ superfamily ligands in the human genome and 42 ligands generated in total, there are so far only seven type I, and six type II receptors have been identified. Formation of diverse receptor complexes leads to ligand specificity and diverse biological responses. However, more interestingly, there are only five R-Smads (*smad1, -2, -3, -5,* and *-8*) characterised for the TGFβ 'canonical' pathway (Derynck and Zhang, 2003; Shi and Massague, 2003) (figure 23).

1.2. 'Canonical' TGFβ signalling pathway; intracellular signalling, R-Smads

The Smads are the only common intracellular signalling molecules for TGFβ 'canonical' signalling pathway. The Mothers against decapentaplegic homology proteins (Smads) are functionally sub-divided into three groups such as receptor Smads (R-Smads; Smad1, -2, -3, -5, and -8), common-Smad (C-Smad; Smad4), and inhibitory Smads (I-Smads; Smad6 and -7). All Smads have a conserved Mad homology 2 (MH2) domain at their C-termini and a less conserved MH1 domain at

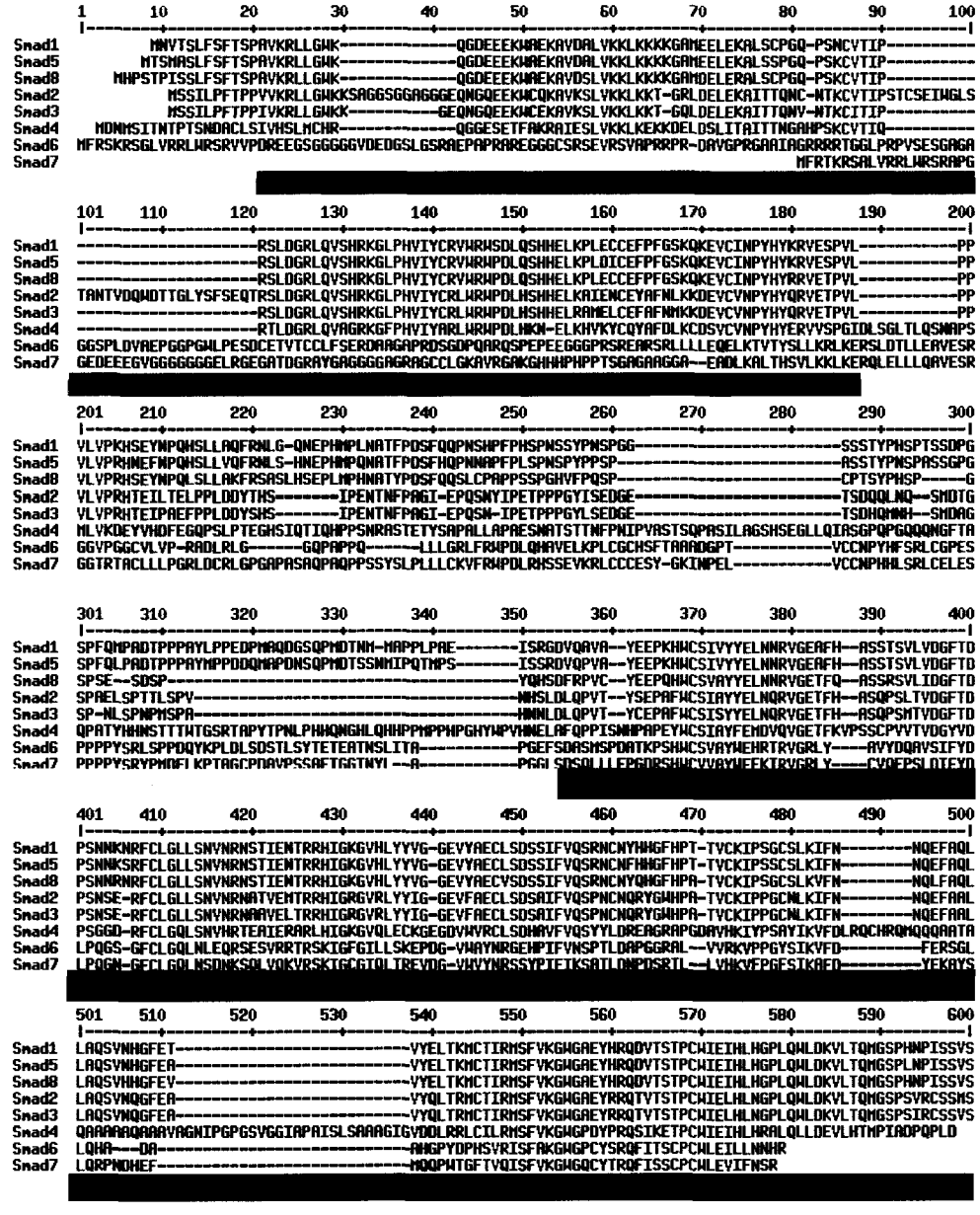


Figure 24. **Amino acid sequence alignment of Smad proteins (mouse)**

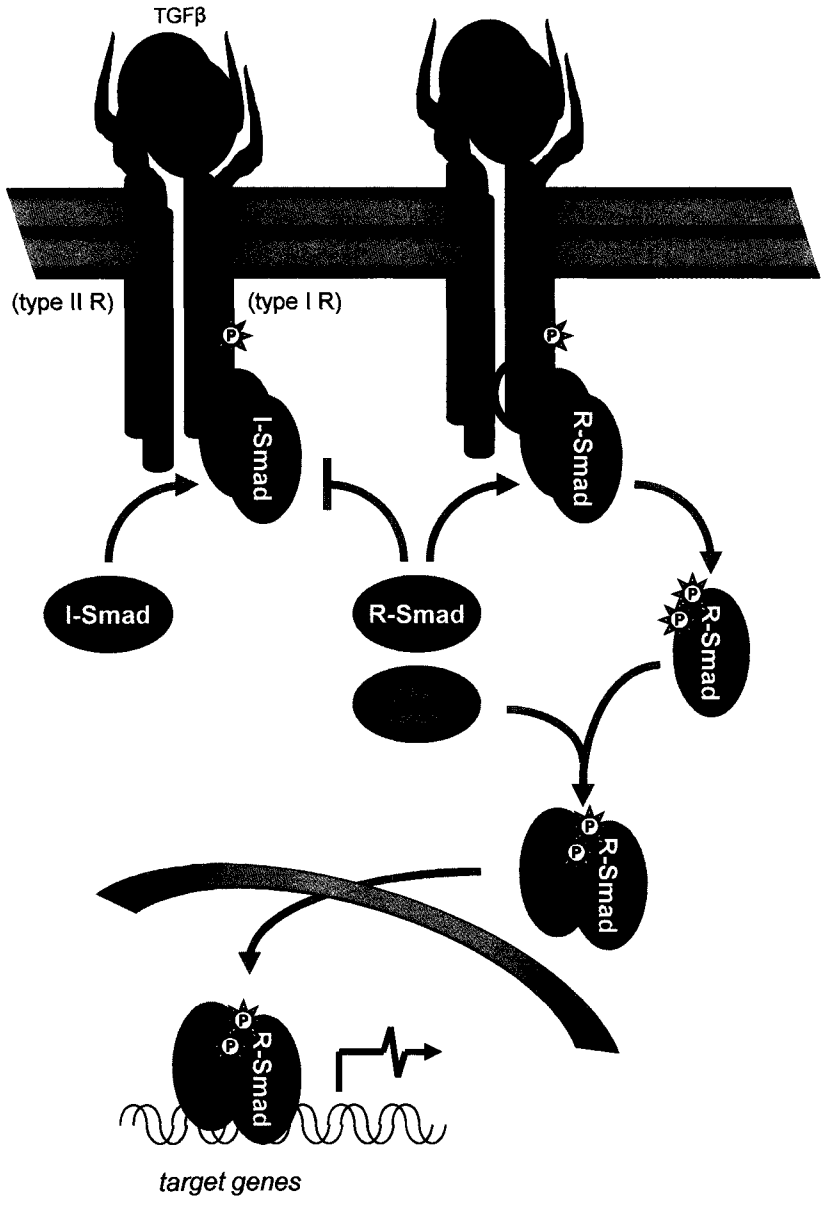


Figure 25. **'Canonical' TGFβ signalling pathway**

their N-termini (Hayashi et al., 1997; Heldin et al., 1997; Massague, 1996; Nakao et al., 1997) (figure 24). R-Smads are recruited to the cytoplasmic tail of the type I receptor, at which both serines in the SXS motif in the C-terminus end of the R-Smads are phosphorylated by the type I receptor. Upon being phosphorylated, the R-Smad dissociates from the receptor due to a conformational change, and associates with Smad4 to form a trimeric complex (two R-Smads and one C-Smad (Smad4); RC-Smad complex) which translocates into the nucleus and regulates target gene expression (Chacko et al., 2004; Jayaraman and Massague, 2000; Kawabata et al., 1998; Lo et al., 1998) (figure 25). Nuclear translocation of the RC-Smad (Smad2/3/4 complex) requires WW domain containing transcription regulator-1/transcriptional co-activator with PDZ-binding motif (TAZ) which also recruits a transcriptional mediator complex to the DNA bound complex (Varelas et al., 2008). On DNA, Smads may form dimers depending on the other interacting proteins (Inman et al., 2002).

1.3. 'Canonical' TGFβ signalling pathway; DNA binding of R-Smad

The consensus common DNA sequence for RC-Smad complex binding is 5'-GTCTAGAC-3'. The MH1 domain of Smad3 interacts with 5'-GTCT-3' and its reverse complement 5'-CAGA-3' (the Smad binding element; SBE) (Shi et al., 1998). However, DNA binding affinity of the RC-Smad complex is very low, so in Smad target promoters, the binding site for the Smad interacting transcription factors are often found adjacent to the SBE (Feng et al., 2000; Qing et al., 2000). Smad3 represses the *c-myc* gene transcription in response to TGFβ by binding to

the 5'-GGCGGG-3' sequence (Frederick et al., 2004). Smad1 can also bind to the 5'-GCCG-3' sequence with high affinity with BMP stimulation (Kim et al., 1997; Kusanagi et al., 2000). Smad4 also binds to 'GC-rich' sequence (Ishida et al., 2000). However, a major isoform of Smad2 is incapable of binding to the DNA due to the disruption of the DNA contacting β-hairpin in the MH1 domain by sequence insertion (figure 24). An alternative spliced variant form of Smad2, which does not have this insert in the MH1 domain, is capable of binding to the DNA with a similar affinity of that of Smad3 (Shi et al., 1998; Yagi et al., 1999).

A variety of sequence specific DNA binding transcription factors interact with Smad proteins physically and functionally. This allows the R-Smads to regulate diverse biological processes.

1.4. 'Canonical' TGFβ signalling pathway; co-activator/co-repressor of R-Smads

R-Smad interacts not only with sequence specific DNA binding transcription factors but also with a large number of transcriptional co-activators or co-repressors. The transcriptional activation properties of R-Smads depend on the interaction with CREB binding protein (CBP)/ p300. The MH2 domain of R-Smads and CBP/p300 interaction requires phosphorylation of the C-terminal SXS motif (Feng et al., 1998). P300 also interacts with Smad3 through its linker region independent of ligand activation (Wang et al., 2005a). The MH2 domain of Smad4 does not recruit CBP/p300, but in the linker, there is a unique 48-amino acid proline-rich sequence (figure 24). Therefore, Smad4 is capable of interacting with p300 and supports transcription. This sequence is termed the Smad4 activation

domain (SAD) and it is required for the recruitment of p300 to RC-Smad complex (de Caestecker et al., 2000). P300/CBP-associated factor (P/CAF) and GCN5 associate with Smad2 and -3 (Itoh et al., 2000), and GCN5 also interacts with Smad1, -5, and -8 (Kahata et al., 2004). For the up-regulation of the Smad2/3 dependent TGFβ response genes in epithelial cells, BRG1 mediated recruitment of SWI/SNF complex to the target promoter through interaction with Smad2/3 is required with exceptions of the *smad7* and *snoN* genes (Xi et al., 2008). ARC, a component of a mediator complex, associates with Smad2, -3, and -4, but not with Smad1 (Kato et al., 2002). TAZ (transcriptional co-activator with PDZ-binding motif/WW domain containing transcription regulator 1 (WWTR1)) interacts with the RC-Smad complex (Smad2/3/4), and it is required for the nuclear-cytoplasmic shuttling of the RC-complex. The TAZ/RC-Smad complex also recruits ARC105 (a mediator complex component) (Varelas et al., 2008). Smad wing for transcriptional activation (Swift), a nuclear BRCT (BRCA1 C-terminal) domain protein, interacts with Smad2 (Shimizu et al., 2001).

Several proto-oncogene products interact with R-Smads and repress their transcriptional transactivation properties. R-Smads and Smad4 interact with c-Ski and SnoN, and they repress TGFβ inducing the $p15^{ink4B}$ and repressing the *c-myc* gene expression. Therefore, TGFβ inducing growth arrest, mediated by Smad2/3 activation, is reversed by the c-Ski and SnoN (Luo, 2004; Sun et al., 1999; Wang et al., 2000; Wotton and Massague, 2001; Wu et al., 2002). C-Ski interferes with the RC-Smad complex formation and also recruits HDACs to the RC-Smad complex through mSin3 or Nuclear receptor co-repressor (N-CoR) (Luo, 2004; Wu et al.,

2002). c-Ski's inhibitory effect can be reversed by the ectopic expression of Ski interacting protein (SKIP), which is co-activator of Smad2/3 (Leong et al., 2001). A zinc finger transcription factor encoding oncogene, *ecotropic viral integration site-1 (evi1)*, also represses R-Smads transcriptional activity through interaction with the MH2 domain of R-Smads and recruits C-terminal Binding Protein (CtBP) (Alliston et al., 2005; Izutsu et al., 2001; Kurokawa et al., 1998). A homeobox transcription factor, TGFβ-induced factor homeobox-1 (TGIF1), interacts with the MH2 domain of R-Smads and recruits mSin3 and CtBP with HDACs, so TGIF inhibits R-Smads mediated transcription (Lo et al., 2001; Melhuish et al., 2001; Wotton et al., 2001; Wotton and Massague, 2001).

2.1. 'Non-canonical' TGFβ signalling pathway

Beside the 'canonical' TGFβ-Smad pathway, TGFβ also activates a variety of signalling pathways independent of R-Smad; 'non-canonical' TGFβ signalling pathways (Bakin et al., 2000; Bhowmick et al., 2001; Derynck and Zhang, 2003; Edlund et al., 2002; Moustakas and Heldin, 2005; Mulder and Morris, 1992; Shibuya et al., 1996; Yamaguchi et al., 1995).

2.1.1. TGFβ activate kinase 1 (TAK1) signalling

TGFβ activated kinase-1 (TAK1), which is MAPK kinase kinase 7 (MKKK7/MAP3K7) (Shibuya et al., 1996; Yamaguchi et al., 1995), might be responsible for the MAPK activation by TGFβ. A recent study showed that TAK1 stably associates with the cytoplasmic tail of TGFβ type I receptor through the GS domain in an unstimulated state. Upon ligand stimulation, TAK1 dissociates from

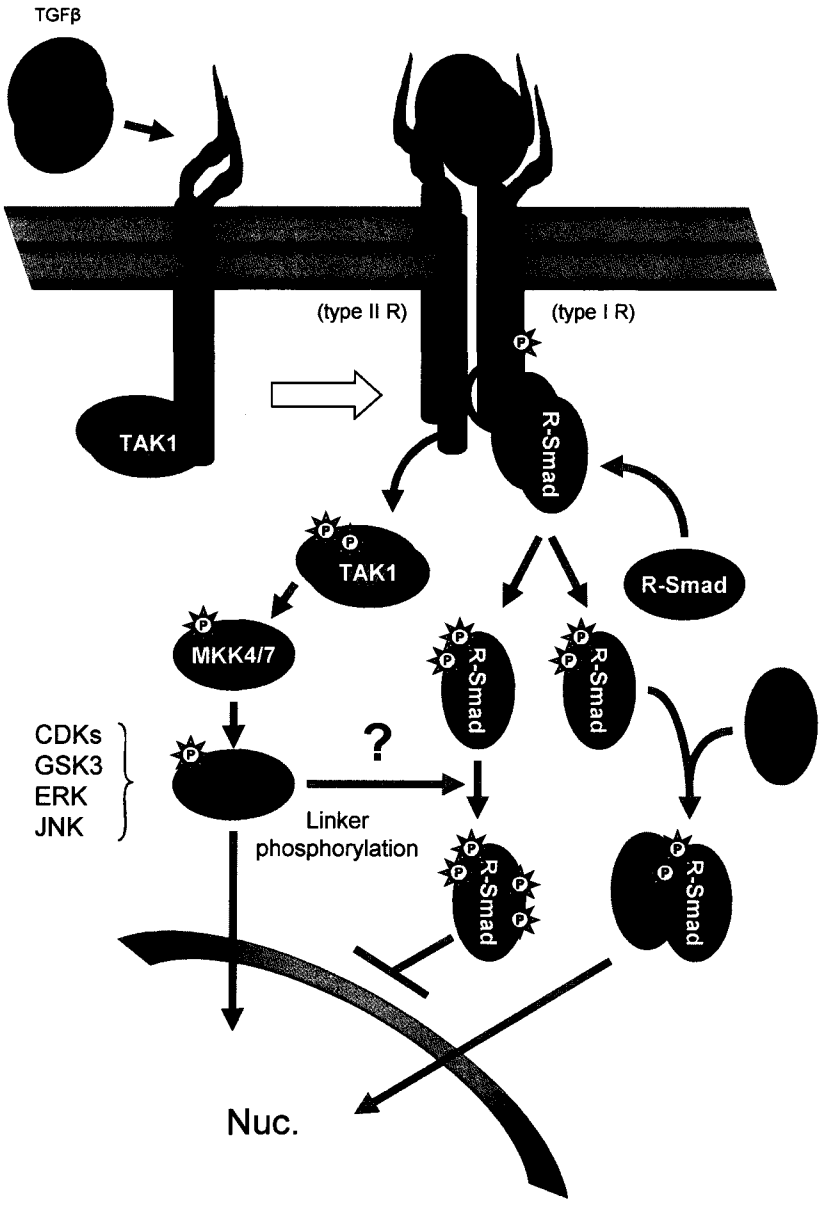


Figure 26. **'Non-canonical' TGFβ signalling; TAK1 activation**

the receptor and auto-phosphorylates (but not by the type I receptor) for activation (Kim et al., 2009) (figure 26). Since TAK1 is an MKKK, it can stimulate the MAPK kinase (MKK) 4/7-JNK and MKK3/6-p38MAPK cascades (Ishitani et al., 1999; Ninomiya-Tsuji et al., 1999). TAK1 plays a crucial role for TGFβ inducing ECM production and fibrosis. Expression of the type I collagen and fibronectin depend on the MKK3-p38MAPK and MKK4-JNK signalling respectively (Hocevar et al., 2005; Zhang et al., 2000a). Increasing and enhancing TAK1 activity in the TAK1 transgenic mice lead to the p38MAPK activation and promote the interstitial fibrosis in myocardium (Zhang et al., 2000a). TGFβ also regulates apoptosis through TAK1 activation (Sayama et al., 2006; Thiefes et al., 2005), and also TAK1 cross-talks to the Wnt signalling (Smit et al., 2004). Interestingly, TAK1 is capable of interacting with all Smad proteins. This interaction is mediated by MH2 domain of the Smad and active kinase domain of TAK1, and inhibits the R-Smads transcriptional activity (Hoffmann et al., 2005). Therefore, activation of TAK1 by TGFβ might be a part of negative feedback loop for the 'canonical' TGFβ pathway.

2.1.2. Mitogen activated protein kinase (MAPK) cascades

TGFβ activates all three branches of mitogen-activated protein kinase (MAPK) pathways, such as c-Jun N-terminal kinase (JNK) (Atfi et al., 1997; Hocevar et al., 1999), p38MAPK (Chin et al., 2001; Hanafusa et al., 1999; Wang et al., 2002), and Extracellular signal regulated kinase (ERK) (Hartsough and Mulder, 1995; Mucsi et al., 1996).

TGFβ induces the *connective tissue growth factor* (*ctgf/ccn2*) gene in the Ras/MEK/ERK signalling dependent manner but independent of R-Smad activation, and CTGF/CCN2 is required for ECM deposition and epithelial tissue repair (re-epithelialization) (Secker et al., 2008). CTGF/CCN2 is also required for TGFβ inducing focal adhesion kinase (FAK)/Akt activation (Shi-wen et al., 2006), and Tumour necrosis factor α (TNFα) inhibits TGFβ induced *ctgf* gene expression (Yu et al., 2009). TGFβ induced apoptosis in the renal tubular epithelial cell (tubular atrophy; due to chronic renal interstitial fibrosis) is independent of Smad2 activation but requires p38MAPK activation (Dai et al., 2003). Either TGFβ or BMP up-regulates the Cyclin dependent kinase (CDK) inhibitors (CKIs) such as cyclin-dependent kinase inhibitor-1B ($p27^{Kip-1}$) and -1A ($p21^{Cip-1}$) through the Ras/MEK/ERK signalling activation in the intestinal epithelial cells. However, one of the targets of this kinase cascade is Smad1 (Hartsough et al., 1996; Hartsough and Mulder, 1995; Mulder and Morris, 1992; Yue et al., 1999). Therefore, the 'non-canonical' signalling pathway may crosstalk to the 'canonical' TGFβ/R-Smad pathway.

2.1.3. Phosphatidylinositol 3-kinase (PI3K)/Akt (Protein kinase B (PKB)) pathway

TGFβ mediated cell migration and epithelial to mesenchymal transition (EMT) are regulated by the RhoA/PI3K/Akt (PKB) signalling pathway. Interestingly, the phosphorylation of Smad2 SXS motif depends on this signalling pathway (Bakin et al., 2000). TGFβ induced destabilisation of the E-cadherin-mediated cell-cell

adhesion is regulated by the phosphorylation of β-catenin through the activation of PI3K, and it is independent of the RC-Smad complex activity (Vogelmann et al., 2005). TGFβ type I receptor physically interacts with PI3K (p85), and PI3K activity is enhanced by the activation of the TGFβ receptor. Interestingly, Smad7 prevents TGFβ inducing PI3K activity (Yi et al., 2005). In human lung fibroblasts, TGFβ induces *α5β3 integrins*, which is required for FAK and c-Src kinase activation. Interestingly, TGFβ activates p38MAPK but not Smad2/3 in a Src family non-receptor tyrosine kinases dependent manner (Pechkovsky et al., 2008). However, in an early stage colon carcinoma cell line (FET/DNRII), TGFβ inhibits the PI3K/Akt/survivin signalling pathway, so loss of TGFβ signalling interferes with apoptosis and promotes carcinogenesis (Wang et al., 2008a).

2.1.4. Rho signalling

The TGFβ 'non-canonical' signalling pathway regulates axon growth. Actin cytoskeleton is regulated by TGFβ, which activates Rho GTPases Rho1 and Rac (Ras-related C3 botulinum toxin substrate (Rho family, small GTP binding protein)), and LIM kinase1 (LIMK1), which require for axon growth (Ng, 2008). TGFβ induction of RhoA/Rho-kinase activity is also required for EMT (Kaartinen et al., 2002). However, although ectopic expression of Smad7 or a dominant negative form of Smad3 (DN-Smad3) silences TGFβ 'canonical' pathway, Smad7 or the DN-Smad3 could not inhibit TGFβ inducing EMT. TGFβ activated RhoA signalling is also not inhibited by Smad7 (Bhowmick et al., 2001). TGFβ induced

RhoA and Cell division cycle 42 (GTP binding protein, 25kDa/Cdc42) activation surprisingly requires Smad7 (Edlund et al., 2004).

Vascular cell adhesion molecule-1 (VCAM1), which is a ligand of α4β1 integrin, inhibits the p190RhoGAP mediating EMT induced by TGFβ (Dokic and Dettman, 2006). Interestingly, the other classes of small GTPases also regulate TGFβ signalling. For example, an internalization of TGFβ receptor complex by endocytosis is regulated by the small GTPases Rab, RalA/Ral-binding protein 1 and Rap2, and Ran GTPase involves in the RC-Smad complex translocation to the nucleus through the nuclear pores (Kardassis et al., 2009). Therefore, there might be crosstalk between TGFβ and small GTPases signalling.

3.1. Cross talk between 'canonical' and 'non-canonical' TGFβ signalling pathway; Effect of linker phosphorylation on R-Smad activity

R-Smads are phosphorylated at both serines in the SXS motif in their C-termini by the type I receptor upon ligand stimulation. In addition to these phosphorylations, R-Smads are also phosphorylated at their linker region, in which there are numbers of S/TP sites (figure 24). These sites are targeted by the proline directed kinases such as Mitogen activated protein kinases (MAPKs) or Cyclin dependent kinases (CDKs). Therefore, 'non-canonical' TGFβ signalling pathway especially through TAK1 activation might target these sites and regulate the R-Smad activity (Derynck and Zhang, 2003; Massague, 2003) (figure 26). In this regard, TAK1 activation might be a part of negative feedback loop for the R-Smads activation by TGFβ. In fact, TGFβ induces phosphorylation of Smad3 at the C-terminus by the type I

receptor and the linker regions by GSK3. Inhibition of GSK3 activity reduces linker phosphorylation, which enhances Smad3 transcriptional activity by the increased interaction with CBP (Millet et al., 2009). Furthermore, the C-terminal tail phosphorylation of the type I TGFβ receptor is a prerequisite for the phosphorylation of the linker regions of Smad3 by GSK3 and CDKs (Wang et al., 2009). Therefore, the membrane localization of the R-Smad is required for the linker phosphorylation. The linker phosphorylation also marks R-Smads for degradation. The phosphorylation of the linker regions increases Pin1 (a peptidylprolyl cis-trans isomerase) interaction with the linker legions of Smad2/3. It further recruits Smad ubiquitin regulatory factor-2 (Smurf2) to the linker regions, and Smurf2 targets the Smad2/3 for ubiquitin mediated degradation (Nakano et al., 2009). Therefore, the phosphorylation of the linker regions of the R-Smads may serve as a negative feedback loop for the R-Smad activation by TGFβ. In addition, TAK1 physically interacts with all R-Smads and inhibits transcriptional activity (Hoffmann et al., 2005). Therefore, TAK1 may negatively feedback TGFβ 'canonical' signalling pathway by multiple mechanisms.

The *furin* gene, which encodes a predominant convertase for the maturation of a number of growth/differentiation factors, is regulated by the TGFβ signalling in an R-Smad dependent manner. Although the phosphorylation sites were not specified, Smad2 phosphorylation and nuclear translocation are enhanced by TGFβ and also mediated by ERK activity in HepG2 cells. Thus, Smad7 and MEK inhibitors interfere with *furin* gene up-regulation by TGFβ (Blanchette et al., 2001a; Blanchette et al., 2001b).

3.1.1. Cross talk by the posttranslational modification of the linker; phosphorylation/sumolylation

R-Smads activity is not only regulated by the phosphorylation at the both serines in the SXS motif at C-terminus but also the phosphorylations at the linker region of R-Smads. The linker region has several S/TP sites, which are targeted by the proline directed kinases such as Mitogen activated protein kinases (MAPKs) or Cyclin dependent kinases (CDKs). However, the effect of the phosphorylation of these sites on the R-Smad activity seems to be context dependent. ERK phosphorylates the linker region of Smad1/2/3 and antagonizing the anti-proliferative affect of TGFβ by inhibiting the nuclear translocation of the RC-Smad complex (Kretzschmar et al., 1997a; Kretzschmar et al., 1999; Kretzschmar et al., 1997b; Pera et al., 2003). The antagonistic relationship between BMP and FGF8/IGF2 for neural induction during embryo development can be explained that Smad1 activation by the BMP signalling inhibited by the linker phosphorylation by the MAPK stimulated by the FGF and IGF (Aubin et al., 2004; Pera et al., 2003). However, the other groups reported that Ras/ERK or JNK inducing phosphorylation in the linker region has no effect or even enhances the nuclear translocation of the RC-Smad complex (de Caestecker et al., 1998; Engel et al., 1999; Lehmann et al., 2000). Furthermore, p38MAPK co-operates with the Rho/Receptor for activated protein kinase C (ROCK) signalling and phosphorylates the serines residues in the linker region of Smad3. These modifications enhance TGFβ mediated Smad dependent down-regulation of the *c-myc* and up-regulation of $p21^{Cip\text{-}1}$ gene expression (Kamaraju and Roberts, 2005). However, in the human kidney epithelial

cell line, ROCK1 physically interacts with the linker region of Smad3 and interferes with Smad3 DNA binding (Okano et al., 2006). The linker region is also phosphorylated by CDKs. CDK2/4 inhibit the Smad2/3 dependent transcription and TGFβ's anti-proliferative effect by the phosphorylation of the linker regions of Smad2/3. However, the target sites of the CDKs are different from that of ERK (Matsuura et al., 2004).

Small C'-terminal domain phosphatases 1, 2, and 3 (SCP1-3) dephosphorylate not only Smad1's C'-terminal tail (SXS) but also the linker region of Smad 1/2/3. This dephosphorylation causes inactivation of Smad1 but enhancement of Smad2/3 activity stimulated by TGFβ (Sapkota et al., 2006). PP2A (Serine/threonine protein phosphatase-2A) interacts with the BMP type II receptor and dephosphorylates the linker region of Smad1 and enhances the nuclear translocation of Smad1 (Bengtsson et al., 2009).

The linker region is not only phosphorylated but also post translationally modified by the other means. For example, the linker region of Smad4 is sumolylated by the E3 ligases such as Protein inhibitor of activated STAT 1 (PIAS1) and PIASxβ upon TGFβ stimulation. Interestingly, p38MAPK not only stabilized PIASxβ protein but also enhanced *pasxβ* gene expression, and consequently p38MAPK activates Smad-dependent transcription by enhancing SUMO-1 (Small ubiquitin-like modifier-1) modification on the linker region of Smad4 (Ohshima and Shimotohno, 2003). PIASy interacts with the MH2 domain of Smad7 and Smad3, and sumoylation of Smad3 by PIASy, in this case, suppresses Smad3 activation by TGFβ (Imoto et al., 2003). In contrast to PIASs, other E3 ubiquitin ligase, Itch (itchy E3 ubiquitin

protein ligase homolog), promotes Smad2 ubiquitination, which facilitates the Smad2/TGFβ receptor complex formation and increases the phosphorylated Smad2 and Smad2 dependent transcription (Bai et al., 2004).

Since Smad4 and R-Smads have a nuclear export signal (NES) in the linker region (Smad1 also has the second NES), the post-translational modification (PTM) of the linker region of R-Smads and Smad4 may regulate sub-cellular localization of the Smad proteins (Xiao et al., 2003). The PTM of the linker region may also affect on the recruitment of p300 since the linker region of Smad4 and Smad3 has the SAD, which is required for the p300 docking (Inman, 2005).

4.1. Functions of the inhibitory Smads (I-Smads); Smad6

Smad6 is an inhibitory Smad (I-Smad) for 'canonical' BMP signalling. Smad6 has a highly conserved MH2 domain, but the N-terminal MH1 domain is divergent to the other Smads. Smad6 interacts with the type I BMP receptors and prevents the R-Smads (Smad1/2 but not Smad3) recruitment to the type I receptors. Consequently, 'canonical' BMP signalling is repressed by Smad6 (Goto et al., 2007; Imamura et al., 1997) (figure 25). This interaction is enhanced by Transducer of ErbB-2.1 (Tob1), which associates with Smad6 at the plasma membrane (Yoshida et al., 2003). However, another group reported that Smad6 has no effect on the phosphorylation of Smad1 by the type I receptor stimulated by the BMP. Smad6 instead physically associates with phosphorylated Smad1 and competes for the RC-Smad complex formation with Smad4 (Hata et al., 1998). In addition, a few mechanisms by which Smad6 inhibits the BMP signalling have been proposed.

First, Smad6, but not Smad7, forms a DNA bound complex with both Homeobox C8 (HoxC8) and HoxA9. Smad6 inhibits interaction of Smad1 with HoxC8, and consequently the BMP signalling (Bai et al., 2000). Smad6 and Smad1 interacts with most of homeobox (Hox) proteins and Smad6 inhibits Hox proteins' transcriptional activation properties (Li et al., 2006b). Second, Smad6 binds DNA and recruits class I, but not class II, HDACs and inhibits gene expression stimulated by BMP (Bai and Cao, 2002). Third, Smad6 also interacts with the N-terminus of the glucocorticoid receptor (GR) through its MH2 domain and suppresses the GR-mediated transcriptional activity *in vitro* by recruiting HDAC3 (Ichijo et al., 2005). Smad6 also recruits CtBP through its linker region which has a CtBP binding motif, PLDLS (Lin et al., 2003). The mutation of the CtBP binding motif in Smad6 causes neutralization of the inhibitory effect of Smad6 on the BMP inducing gene expression, for example the *id1* gene (Lin et al., 2003). Fourth, Smad6 also inhibits 'non-canonical' BMP signalling pathway though interaction with TAK1. Smad6 inhibits TAK1/p38MAPK activation by BMP2 and consequently BMP2 inducing apoptosis in mouse hybridoma MH60 cells (Kimura et al., 2000). Finally, Osteoblast differentiation induced by BMPs and exogenous expression of an active BMP type I receptor in a multi-potent mesenchymal cell, C2C12, is inhibited by Smad6 and the other I-Smad, Smad7 (Fujii et al., 1999; Valcourt et al., 2002). Smad6 reverses BMP2 but not Activin induced growth arrest, and Smad7 antagonizes Activin's but not BMP2's anti-proliferative effect (Ishisaki et al., 1999).

4.1.1. Smad6 gene expression

Expression of the *smad6* gene is remarkably restricted to the developing heart, eyes, and limbs during embryo development, and Smad6 expression is induced by BMP2 and down-regulated Noggin, an antagonist of BMP (Vargesson and Laufer, 2009; Yamada et al., 1999). In agreement with this observation, the *smad6*-/- mouse has multiple cardiovascular abnormalities (Galvin et al., 2000).

The promoter/enhancer region of the *smad6* gene has a Smad (Smad1/5) binding site, which responds to the BMP stimulation and is termed as proximal BMP-responsive element (PBE) (Nakanishi et al., 2000). OAZ/Zinc finger protein-423, a critical co-activator of Smad1/4, enhances *smad6* expression upon BMP stimulation (Ku et al., 2006). BMP2 also induces *runt-related transcription factor-2* (*runx2*), which binds to the *smad6* promoter and activates the *smad6 gene* expression. Smad1 and Smurf1 are also recruited to the PBE and regulate the *smad6* gene transcription positively and negatively (Wang et al., 2007). Interestingly, TGFβ antagonizes the BMP signalling through inducing *smad6* expression (Shen et al., 2007). It suggests that there is a cross-talk between the TGFβ and BMP signalling through I-Smads.

Finally, besides *smad6* gene regulation, Smad6 protein activity is inhibited by Associated molecule with the SH3 domain of signal transducing adaptor molecule ((SH3 domain and ITAM motif) (STAM)) (AMSH). AMSH interacts with Smad6 in the cytoplasm and sequestrates Smad6 from other binging partners (Itoh et al., 2001).

4.2. Functions of the inhibitory Smads (I-Smads); Smad7

Smad7 is the other I-Smad. Structurally, Smad7 is very similar to Smad6; Smad7 has a highly conserved MH2 domain at its C-terminus but less conserved MH1 domain at its N'-terminus (figure 24). Smad7 inhibits the TGFβ/Nodal signalling by interference with the R-Smads (Smad2/3) phosphorylation by the type I receptors (Hayashi et al., 1997). Smad7 also interferes with the type I BMP receptor mediated R-Smad phosphorylation (Souchelnytskyi et al., 1998) (figure 25). Serine/threonine kinase receptor associated protein (STRAP), which associates with both the type I and II TGFβ receptors, co-operates with Smad7, but not Smad6, to interfere with the access of Smad2/3 to the receptor. The four basic amino acids in the C-terminus of Smad7 protein are critical for the association with the type I receptor (Datta et al., 1998; Datta and Moses, 2000; Mochizuki et al., 2004). STRAP also associates with Pyruvate dehydrogenase kinase-1 (PDK1). STRAP/PDK1 complex co-operates with Smad7 and enhances the inhibition of TGFβ 'canonical' signalling (Seong et al., 2005) (figure 27A). PDK1 interacts with Smad2, -3, -4, and -7, and this interaction enhances PDK1 kinase activity but inhibits R-Smad transcriptional activation properties. Therefore, PDK1 and Smad7 may co-operate to inhibit the TGFβ 'canonical' signalling pathway by two mechanisms (Seong et al., 2007) (figure 27A). The *salt-inducible kinase* (*sik*), which is induced by TGFβ in a Smad4 dependent manner, also physically associates with Smad7 and enhances degradation of the type I TGFβ receptor through a complex formation with Smad7/SIK/type I receptor. Therefore, SIK may participate in the negative feedback of TGFβ 'canonical' signalling (Kowanetz et al., 2008) (figure 27B).

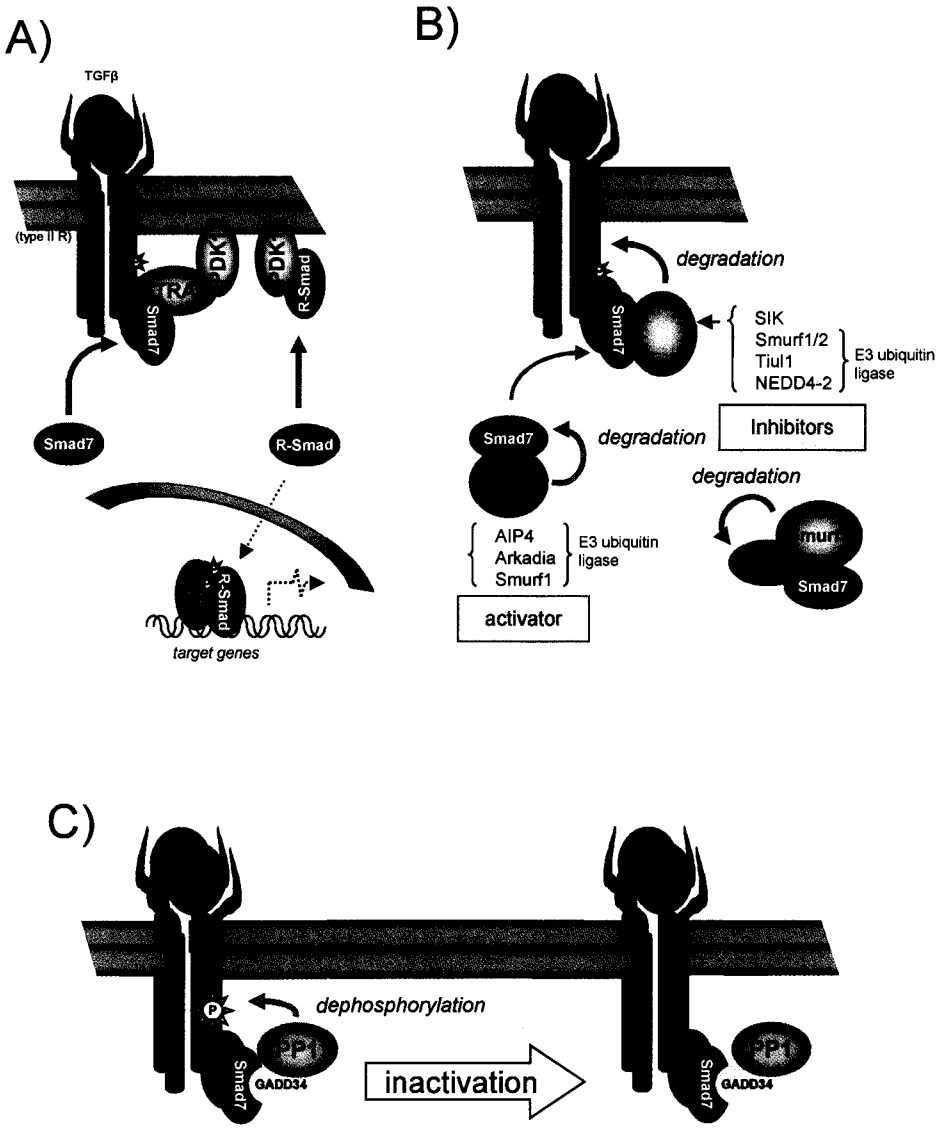


Figure 27. **Smad7 mediated regulation of TGFβ signalling**

However, the most established inhibitors for Smad7 dependent inhibition of TGFβ signalling pathway are E3 ubiquitin ligases. For example, Smurf interacts with the type I receptor through I-Smads and promotes receptor degradation. Smurf interacts with an evolutionarily conserved PY motif in the linker region of Smad7 (Chong et al., 2006). Therefore, Smad7 works as a bridging protein between the TGFβ receptor and Smurf1 (Murakami et al., 2003) (figure 27B). Smurf2 also binds Smad7 and targets the TGFβ type I receptor for degradation (Ebisawa et al., 2001; Kavsak et al., 2000; Ogunjimi et al., 2005). Furthermore, E3 ubiquitin ligases such as Tiul1 (TGIF (TGFβ induced factor homeobox 1) interacting ubiquitin ligase-1) and NEDD4-2 (Neural precursor cell expressed, developmentally down-regulated 4-2), inhibit TGFβ signalling in a similar manner (Kuratomi et al., 2005; Seo et al., 2004) (figure 27B). Atrophin 1-interacting protein 4 (AIP4), as an E3 ubiquitin ligase, also interacts with Smad7, but AIP4 targets Smad7 but not the TGFβ receptor for ubiquitin mediated degradation. Therefore, AIP4 activates the TGFβ pathway by removing Smad7 (Lallemand et al., 2005) (figure 27B). Smad7 and Smad6 also enhance degradation of Smad4 in co-operation with Smurf1. Smad7 co-localizes with Smad4 in the cytoplasm, and this co-localization brings Smurf1 to Smad4. Consequently, Smurf1 targets Smad4 for ubiquitin mediated degradation, and Smad7 inhibits the TGFβ pathway through reduction of C-Smad (Moren et al., 2005) (figure 27B). Smad7 itself is also targeted by the TGFβ induced Smurf1 mediated ubiquitin-proteolysis. HAT, p300, interacts with Smad7 and acetylates two lysine residues at the N'-terminus of Smad7. Since these lysines are also

targeted by the ubiquitination by Smurf1, p300 protects Smad7 from TGFβ induced degradation by the competition for the PTM of these lysines with Smurf1 (Gronroos et al., 2002) (figure 28B). Another E3 ubiquitin ligases positively regulate TGFβ 'canonical' pathway by interacting with Smad7 is Arkadia, which was originally identified as a Nodal signalling enhancer (Episkopou et al., 2001; Niederlander et al., 2001), physically interacts with Smad7 but not the TGFβ receptor and leads to poly-ubiquitination of Smad7. Consequently, poly-ubiquitinated Smad7 by Arkadia is targeted for degradation. TGFβ/BMP signalling is therefore enhanced by ectopic expression of Arkadia, and Arkadia is required for TGFβ/BMP induced R-Smad activation (figure 27B). Interestingly, since Arkadia expression is down-regulated by TGFβ, Arkadia may be part of a negative feedback loop for TGFβ signalling (Koinuma et al., 2003). The Arkadia/Smad7 interaction is scaffolded by Axin, and Axin enhances Smad7 degradation mediated by Arkadia and potentiates TGFβ signalling pathway (Liu et al., 2006). Arkadia also enhances degradation of the co-repressors for R-Smads, SnoN and c-Ski, by ubiquitination which also potentiates TGFβ 'canonical' signalling pathway (Nagano et al., 2007).

Furthermore, Smad7 recruits Growth arrest and DNA damage-34 (GADD34) protein to the receptor. Since GADD34 is a regulatory subunit of the protein phosphatase-1 (PP1), the type I TGFβ receptor is dephosphorylated and inactivated by the PP1 (Shi et al., 2004) (figure 27C). Smad7 interacts with TAK1-associated Binding Protein2 and -3 (TAB2/3) and interferes with the TAK1 activation by TGFβ. It causes inactivation of NF-κB, which is a key transcriptional regulator for pro-inflammatory cytokine induction by TNFα (Hong et al., 2007). Ectopic

expression of Smad7 can also interfere with Rho activation, which is potential down-stream target of TGFβ, and cytoskeletal reorganization by TGFβ in Swiss 3T3 fibroblasts (Vardouli et al., 2005).

4.2.1. Negative targets of Smad7

In support of the inhibitory role of Smad7 on the TGFβ 'canonical' signalling pathway, there are a few examples in which Smad7 negatively regulates R-Smad dependent TGFβ target genes. For example, the *cyclin-dependent kinase inhibitor-1A* ($p21^{Cip-1}$) gene induction by activated Smad2/3 by TGFβ and trans-acting transcription factor 1 (Sp1) can be inhibited by ectopic expression of Smad7 (Pardali et al., 2000). Smad7 also inhibits Activin A-induced expression of $p21^{Cip-1}$ and hypo-phosphorylation of retinoblastoma protein (Rb) and reverses anti-proliferative effect and apoptosis induced by Activin A in the B lineage cells (HS-72) (Ishisaki et al., 1998). Smad7 inhibits the MEKK1, but not MEK1 or TAK1, induced Smad2 activation (Brown et al., 1999). MEKK1, but not MEK1 or TAK1, mediated enhancement of the transcriptional activation properties of Smad2 and the nuclear translocation of Smad2/4 are inhibited by ectopic expression of Smad7 (Brown et al., 1999).

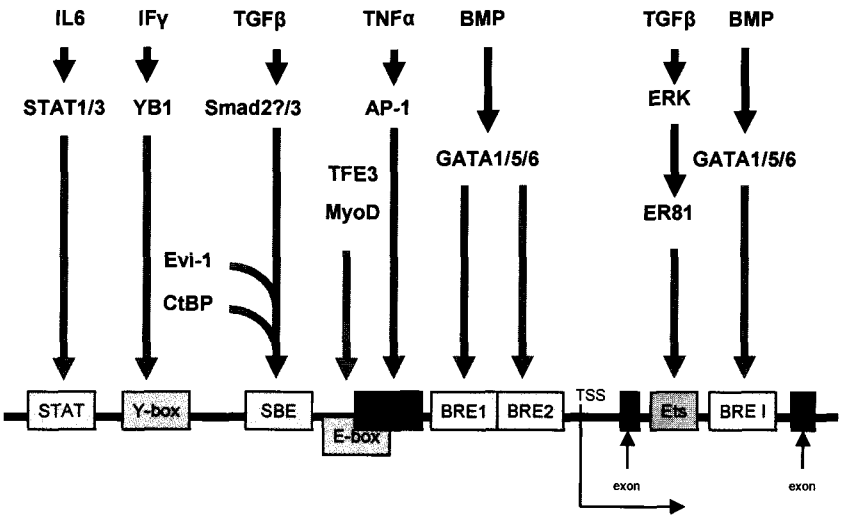
4.2.2. Expression of the Smad7 gene

Smad7 expression is up-regulated by TGFβ stimulation. Therefore, Smad7 forms a negative feedback loop for 'canonical' TGFβ signalling pathway (Nakao et al., 1997). The promoter of the *smad7* gene has an SBE, and it responds to TGFβ and an ectopic expression of Smad3 (Denissova et al., 2000; Nagarajan et al., 1999)

(figure 28A). Since R-Smad often co-operates with other classes of transcription factor for the regulation of its target genes, the *smad7* gene is also co-regulated by a variety of transcriptional regulators in an R-Smad dependent and independent manner. TGFβ activated Sp1 and AP1 co-operate with Smad2/3 and induces the *smad7* gene transcription (Brodin et al., 2000). Although the SBE bound protein complex contains Smad2/3/4, genetic targeting study of the *smad2, -3*, or *-4* gene indicated that Smad3 and Smad4, but not Smad2, are required for TGFβ inducing the *smad7* gene expression (von Gersdorff et al., 2000). Ski, a co-repressor for Smad4, negatively regulates the *smad7* gene transcription depending on the binding of the Smad4 on the SBE (Denissova and Liu, 2004). Evi-1 and CtBP also repress the *smad7* gene transcription by interacting with the MH2 domain of the R-Smads (Alliston et al., 2005). A bHLH transcription factor, Transcription factor binding to IGHM enhancer-3 (TFE3), and Smad3 synergistically induces the *smad7* gene expression transcriptionally through binding to an E-box and an adjacent SBE respectively with TGFβ stimulation (Hua et al., 2000; Stopa et al., 2000). BMP also induces the *smad7* gene transcription through multiple BMP response elements (BREs). BRE1 and -2 are located in the 5'-regulatory region of the *smad7* gene, and the third one is located in the first intron (I-BRE). The BRE1 and -2 activities are regulated by Smad1/4, and the I-BRE is activated by GATA binding protein-1 (GATA1), -5, or -6 factors (Benchabane and Wrana, 2003). JAK1/STAT1 (Ulloa et al., 1999) and NF-κB/RelA (Bitzer et al., 2000) are activated by Interferon-γ (IFNγ) and antagonises TGFβ action by the up-regulation of the *smad7* gene. Furthermore norepinephrine, a α1-adrenergic agonist, induced the *smad7* gene expression is

mediated by activation of NF-κB in hepatocytes (Kanamaru et al., 2001). In osteosarcoma Saos2 osteoblasts, NF-κB is constitutively activated, and this leads to a higher expression level of Smad7 which prevents BMP2 inducing osteoblast differentiation (Eliseev et al., 2006). However, another group reported that an ectopic expression NF-κB/p65 or TNFα activated NF-κB inhibits the *smad7* gene expression. Since p300 expression relieves the NF-κB's inhibitory effect on the *smad7* gene expression, the competition between Smad2/3 and NF-κB for p300 binding has been suggested (Nagarajan et al., 2000). However, another study showed that TNFα inhibits TGFβ target gene expression by the activation of AP1 but not Smad7, and the *smad7* gene expression levels are not affected by TGFβ stimulation (Verrecchia et al., 2000). In breast, endometrial, and ovarian cancer cell lines, the *smad7* gene expression was up-regulated by HER/Neu (v-erb-b2 erythroblastic leukemia viral oncogene homolog, neuro/glioblastoma derived oncogene homolog) and ER81 (ets variant-1) through binging to two Ets binding sites in the *smad7* promoter, and either ERK or TAK1/p38MAPK activity is required (Dowdy et al., 2003) (figure 28A). Furthermore, the *smad7* gene expression is an immediate-early gene target of TGFβ in fibroblasts (Mori et al., 2000). However, Ultraviolet (UV) irradiation blocks TGFβ 'canonical' pathway through the degradation of the type II receptor. In addition, the *smad7*gene transcription is up-regulated by UV irradiation independent of the TGFβ signalling but depending on the AP1 site bound c-Jun and c-Fos (Quan et al., 2001, 2005). It was also reported that IL-6 family cytokines up-regulate the *smad7* gene transcription by activation of STAT3 through gp130 (Jenkins et al., 2005). IL-7

A)



B)

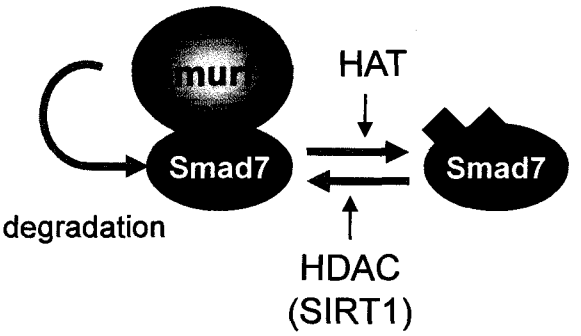


Figure 28. **Smad7 expression** A) transcriptional regulation of the *smad7* gene. (Blue for a negative regulator, Red for positive regulator) B) degradation/stability of Smad7 protein by HDAC/HAT.

protects pulmonary fibrosis induced by TGFβ through up-regulation of the *smad7* gene expression via activation of the JAK1/STAT1 signalling pathway (Huang et al., 2002). Interferonγ (IFγ) can induce the *smad7* gene transcriptionally through JAK1/Y-box protein-1 (YB1), which binds a bona fide binding site in the smad7 promoter (Dooley et al., 2006) (figure 28A).

Smad7 is also regulated by the PTM. Acetylation of two lysine residues in Smad7 protein controls its stability. Acetylation by p300 increases Smad7 stability by preventing the Smurf mediated ubiquitin proteosomal degradation induced by TGFβ, and deacetylation by HDACs enhances the Smad7 ubiquitination (Simonsson et al., 2005). SIRT1, a class III HDAC, interacts with N'-terminus of Smad7 and deacetylates these lysines and facilitates Smurf1 binding to the Smad7 and enhances Smad7 degradation (Kume et al., 2007) (figure 28B). Therefore, Smad7 expression is regulated at the transcriptional level and also by the PTMs.

4.2.3. Sub-cellular localization of Smad7

As a TGFβ 'canonical' signalling pathway inhibitor and a part of a negative feedback loop, Smad7 needs to be localized in the cytoplasm but not in the nucleus. Therefore, the regulation of Smad7 sub-cellular localization is critical for its inhibitory role. Smad7 resides in the both cytoplasm and nucleus, but upon TGFβ stimulation, Smad7 is exported from the nucleus and localized in the cytoplasm. A deletion mutant form of Smad7, which lacks C'-terminal MH2 domain, is mainly localized in the cytoplasm independent of TGFβ stimulation. Therefore, the C-terminus is required for nuclear translocation of Smad7 (Itoh et al., 1998).

However, it has also shown that N-terminal of Smad7 controls its sub-cellular localization, and a chimera of the N'-terminus Smad7 and the MH2 domain of Smad6 more efficiently inhibits TGFβ 'canonical' signalling (Hanyu et al., 2001). However, another group found that TGFβ has no effect on the Smad7 sub-cellular localization in mink lung epithelial (Mv1Lu) cells but the type of cell culture surface does affect it. Smad7 mainly localized in the cytoplasm if the cells were cultured on plastic or on fibronectin-coated glass plates. In contrast, if the cells are on glass, most of the Smad7 protein localizes in the nucleus (Zhu et al., 1999).

A protein-protein interaction controls Smad7 sub-cellular localization. Smurf1 interacts with and ubiquitinates Smad7, and the ubiquitinated Smad7/Smurf1 complex translocates to the cytoplasm (Ebisawa et al., 2001). The nuclear export of the Smad7/Smurf1 complex is mediated by a nuclear export receptor, exportin-1 (CRM1), and C-terminal nuclear export signal (NES) of Smurf1 is required for this CRM1 dependent nuclear export of the Smad7/Smurf complex (Tajima et al., 2003). The N-terminal conserved-2 domain of Smurf1 requires for membrane localization of the Smad7/Smurf1 complex but not association with Smad7 (Suzuki et al., 2002) (figure 29).

Similarly, WWR1/Tiul-1 associates with Smad7 and co-translocates to the cytoplasm with Smad7, and enhances Smad7 and the TGFβ receptor complex formation. Therefore, functionally, WWR1/Tiul-1 inhibits TGFβ 'canonical' pathway through enhancement of the cytoplasmic localization of Smad7 (figure 29). WWR1/Tiul-1 cold not interact with R-Smads (Komuro et al., 2004), but Smad2 was reported to associate with Tiul-1 by another group (Seo et al., 2004). PDK1

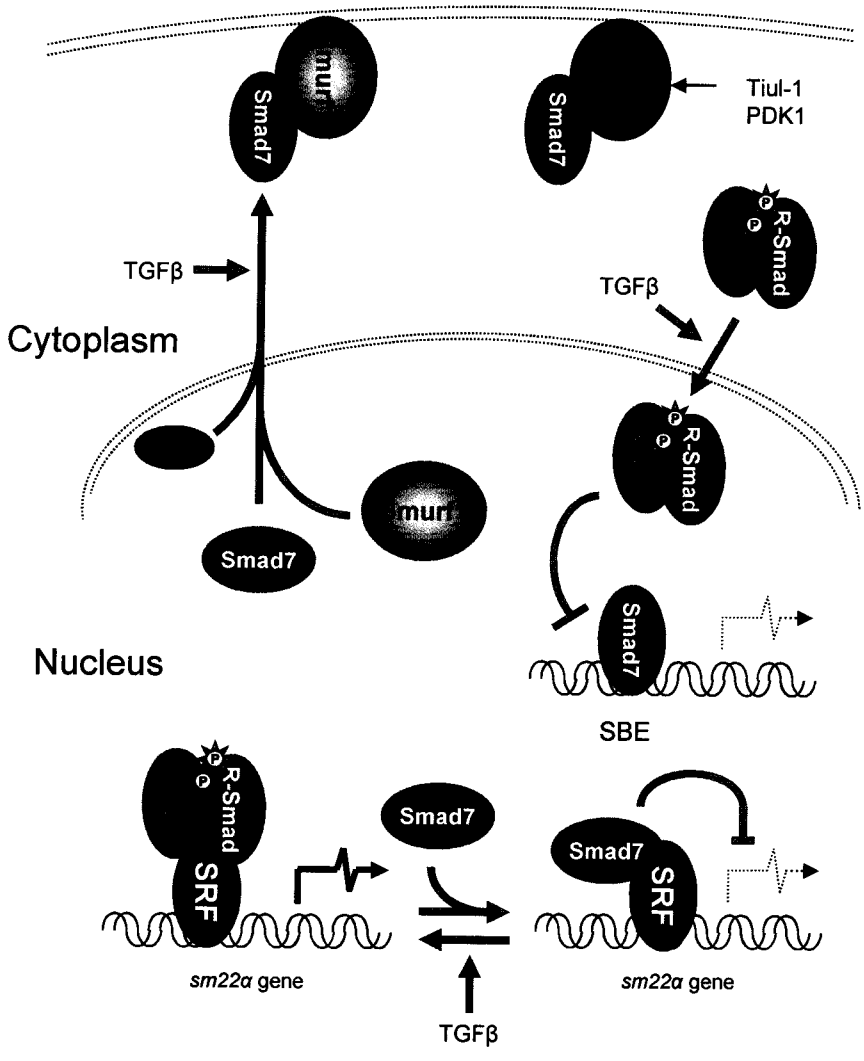


Figure 29. **Sub-cellular localization of Smad7**

also interacts with Smad2, -3, -4, and -7 with its pleckstrin homology (PH) domain. Interestingly, upon stimulation of TGFβ, PDK1 prevents nuclear translocation of Smad2, -3, and -4, and surprisingly inhibits the nuclear export of Smad7 (Seong et al., 2007). COP9 constitutive photomorphogenic homolog subunit 5 (Jab1/CSN5), a subunit of the Constitutive photomorphogenic-9 (COP9) signalosome complex, interacts with Smad7. Upon complex formation, it translocates to the cytoplasm from the nucleus, and then the Smad7 is targeted by degradation (Kim et al., 2004). Since Smad7 is an important inhibitory molecule for TGFβ 'canonical' signalling pathway, these molecules, which regulate Smad7 sub-cellular localization, potentiate or de-sensitize TGFβ signalling. Therefore, it provides the cells to regulate TGFβ signalling pathway by different way other than manipulating the 'core' components of this pathway.

4.2.4. Nuclear function of Smad7

In addition to the established 'canonical' role for TGFβ signalling inhibitor in the cytoplasm, role of Smad7 in the nucleus has been speculated. Smad7 associates with the class I HDAC in the nucleus (Bai and Cao, 2002) and sirtuin (silent mating type information regulation-2) homolog-1 (SIRT1), a class III HDAC (Kume et al., 2007). Smad7 binds to the SBE through its MH2 domain in the *plasminogen activator inhibitor-1* (*pai1*) gene promoter. On the SBE, Smad7 prevents RC-Smad DNA binding to the SBE, therefore, inhibits the TGFβ induced R-Smad dependent transcription of the *pai1* gene (Zhang et al., 2007a). Smad7 can be phosphorylated at serine 249 independent of TGFβ stimulation, and this phosphorylation has no

effect on the ability of Smad7 to inhibit the TGFβ/BMP7 'canonical' signalling but has an effect on transcriptional potential of Smad7 without changing its stability or subcellular localization (Pulaski et al., 2001). Finally, TGFβ induces expression of the *transgelin/smooth muscle 22 protein* (*tagln/sm22α*) gene transcriptionally through enhancing the SRF DNA binding to the promoter. Smad7, in the nucleus, physically interacts with SRF, and this interaction is regulated by TGFβ negatively (Camoretti-Mercado et al., 2006) (figure 29). Therefore, Smad7 may have a nuclear function beside the inhibition of 'canonical' TGFβ signalling pathway at the plasma membrane receptor level.

4.2.5. Biological function of Smad7

In addition to a large number of studies that indicate Smad7 plays biological roles antagonizing the TGFβ signalling (Hayashi et al., 1997; Nakao et al., 1997; Whitman, 1997), other studies including reports from our laboratory suggest that Smad7 may have physiological roles independent of its inhibitory effect on the TGFβ signalling (Kollias et al., 2006; Mazars et al., 2001; Pulaski et al., 2001). Smad7 and Smad6 inhibit BMP2 induced chondrocytic expression and osteoblastic differentiation as inhibitors of the BMP pathway (Valcourt et al., 2002). Interestingly, TGFβ stimulation or ectopic Smad3 and Smad2 expression inhibit adipocyte differentiation. However, ectopic expression of Smad7 and Smad6 also prevent adipogenesis even though they are inhibitors of the 'canonical' TGFβ/BMP signalling pathway (Choy et al., 2000). Similarly, an ectopic expression of Smad7 interferes with the activation of R-Smads (Smad1/5) by the BMP or Activin type I

receptors and the BMP6 inducing osteoblast and chondrogenic differentiation of C2C12 (Fujii et al., 1999). DnaJ (Hsp40) homolog, subfamily A, member 3 (DNAJA3/Tid1) interacts with MH2 domain of Smad7 and blocks the dorsalizing and BMP-dependent regulatory activity of Smad7 in developing *Xenopus* embryos (Torregroza and Evans, 2006). The mouse has the expression cassette of the *smad7* but not *samd6* gene. Surprisingly, Smad7, but not Smad6, down-regulates the phosphorylation of p38MAPK and the chondrocyte differentiation induced by BMP (Iwai et al., 2008). Epithelial-mesenchymal transition (EMT), which is required for the normal tissue patterning and also carcinoma invasiveness, is induced by TGFβ/Activin, but not BMP members of TGFβ superfamily in normal epithelial cells. Ectopic expression of Smad7 prevents the EMT by TGFβ/Activin (Valcourt et al., 2005). In agreement with above observations, single nucleotide polymorphisms (SNPs) in the *smad7* gene were identified in the samples of 940 individuals with familial colorectal tumours (Broderick et al., 2007). Smad7 also enhances degradation of β-catenin and antagonizes the Wnt signalling and interferes with the function of the keratinocytes (Han et al., 2006). Furthermore, Smad7 physically interacts with MyoD and promotes skeletal muscle differentiation independent of its inhibitory effect on the TGFβ 'canonical' signalling pathway (Kollias et al., 2006).

4.2.5.1. Role of Smad7 in embryonic development

BMP inhibits neural induction, and inhibition of this signalling is required for the neural marker gene expression. Therefore, a role of I-Smads in this process has been speculated (Chang and Harland, 2007). The BMP/ALK2/R-Smad pathway

regulates cardiac (left-right) orientation, and Smad7 and Smad1 are the down-stream targets of BMP signalling (Ramsdell and Yost, 1999). In embryos expressing a mutated form of Smad7 (deletion of the MH2 domain), heart development is defective, and most embryos died pre-natally due to multiple defects in cardiovascular development. This indicates that Smad7 is essential for the development and function of the heart *in vivo* (Chen et al., 2009a). In the developing limb buds in a chick embryo, Smad7 and -6 are expressed in the regions expressing BMPs. In the limb mesenchyme, BMP signals may regulate Smad7 and -6 expression (Vargesson and Laufer, 2009). Mis-expression of Smad7, but not Smad6, in pre-chondrogenic cells causes inhibition of chondrocyte differentiation through down-regulation of p38MAPK, which is normally activated by BMP (Iwai et al., 2008).

4.2.5.2. Pathophysiological role of Smad7

Scleroderma is a chronic systemic disease that leads to fibrosis of affected organs. Affected tissue shows hyperactive TGFβ/Smad2/3 activity and reduced expression of Smad7. Ectopic expression of Smad7 reverses this pathological chronical activation of TGFβ signalling (Dong et al., 2002). In a progressive form of tubulointerstitial fibrosis, both Smurf1 and -2 are up-regulated, and their target, Smad7 protein, but not the mRNA level, is reduced progressively, and nuclear phosphorylated Smad2 and -3 are increased (Fukasawa et al., 2004). In contrast, in inflammatory bowel disease (IBD) mucosa and purified mucosal T cells, Smad7 expression is up-regulated, and pro-inflammatory cytokines are chronically

produced. TGFβ signalling restricts production of these cytokines in the unaffected tissues, and the inhibition of Smad7 reduces the inflammatory reaction in the IBD (Monteleone et al., 2001). Activation of NF-κB, which is a key transcription factor for up-regulation of pro-inflammatory cytokines, is a negative down-stream target of TGFβ signalling pathway. In the IBD gut inflammation, TGFβ could not block NF-κB activation due to high level of the Smad7 expression (Monteleone et al., 2004). However, another study showed that TGFβ induced Smad7 physically interacts with TAB2/3, which is required for efficient activation of TAK1. Since TAK1 activates NF-κB, TGFβ repressed TNFα induced inflammatory responses by up-regulation of the *smad7* gene (Hong et al., 2007).

In the development of Alzheimer disease, progressive accumulation of the amyloidβ peptide in the brain is crucial. TGFβ plays a role for the clearance of accumulated amyloidβ peptide by up-regulation of Matrix metalloproteinase-2 (MMP2). Accumulation of the amyloidβ peptide 1-42 leads to up-regulation of the *smad7* gene transcription and down-regulation of MMP2 production, which may cause further accumulation of amyloidβ peptide and silencing TGFβ 'canonical' signalling pathway by up-regulation of the *smad7* gene (Lee et al., 2005a). Smad7 also negatively regulates TGFβ induced collagen production in skin fibroblasts. UV irradiation up-regulates the *smad7* gene transcription though activation of c-Jun and c-Fos, and collagen production in the connective tissue maintained by TGFβ is reduced (Quan et al., 2005). TGFβ plays a major role for fibrosis in diseases such as diabetic nephropathy. CTGF/CCN2 is a down-stream effecter of TGFβ signalling in the fibrosis (Secker et al., 2008), and CTGF enhances the TGFβ 'canonical'

pathway by transcriptional suppression of the *smad7* gene through induction of the transcription factor *kruppel-like factor-10* (*klf10/tieg1*) (Wahab et al., 2005). Conversely, the *smad7* gene induced by IFγ transcriptionally through JAK1/YB1 pathway inhibits TGFβ induced collagen expression. YB1 also directly inhibits collagen (*col1A2*) expression as a transcriptional repressor, but Smad7 also plays a role in the IFγ's anti-fibrotic effect (Dooley et al., 2006). For the skin wound healing, re-epithelialization is essential, and TGFβ signalling plays important roles (Hua et al., 1998). During the re-epithelialization, keratinocyte α3β1 integrin is up-regulated. Surprisingly, the role of α3β1 integrin for keratinocyte migration is not essential, but blockade of Smad7 is required for the re-epithelialization (Reynolds et al., 2008). Smad7 interacts with Axin, and this interaction excludes GSK3β and β-catenin from the Axin scaffolding. Consequently, Smad7 protects β-catenin from being phosphorylated by GSK3β and following degradation. The protected β-catenin forms a complex with E-cadherin instead of translocates into the nucleus. Therefore, Smad7 regulates the cell-cell adhesion through the modulation of β-catenin (Tang et al., 2008).

Thus, maintenance of appropriate Smad7 level is a key regulatory event to control TGFβ signalling pathway in the several different tissues.

4.2.5.3. Role of Smad7 in apoptosis

TGFβ stimulation causes apoptosis, and inhibition of Smad7 expression and, surprisingly, negates TGFβ induced apoptosis in some cell lines. Therefore, TGFβ induces apoptosis depending on Smad7 expression, but not activation of R-Smads

in some cases (Landstrom et al., 2000). Ectopic expression of Smad7 stimulates MKK4/JNK signalling cascade which does not affect on R-Smads activity and induces cell-death (Mazars et al., 2001). In progressive glomeruloscrosis, podocytes are being eliminated by the TGFβ mediated apoptosis, which depends on p38MAPK and Caspase-3 activation. Interestingly, Smad7 also stimulates apoptosis independent of TGFβ signalling through the interference with the nuclear translocation and activation of NF-κB, which is a survival factor in podocytes (Schiffer et al., 2001). Similarly, TGFβ or ectopic expression of Smad7 induces apoptosis in renal glomerular mesangial cells. This apoptotic response is attenuated by the enhanced degradation of Smad7 through deacetylation by SIRT1 and subsequent Smurf1 binding to Smad7 (Kume et al., 2007). Moreover, either ectopic expression of Smad7 or TGFβ stimulation induces apoptosis in human prostate cancer PC-3U cells in a p38MAPK dependent manner. Smad7 surprisingly plays a scaffolding role for the TAK1/MKK3/p38MAPK kinase cascade. Therefore, a key effecter of the induction of apoptosis by TGFβ might be Smad7 (Edlund et al., 2003). In addition, TGFβ does crosstalk to Wnt signalling. β-catenin and LEF1/TCF form a complex with Smad7 in response to TGFβ. TGFβ increases nuclear β-catenin and LEF1/TCF and activity of p38MAPK and Akt/PKB in a Smad7 dependent manner, and apoptosis in PC-3U cells and human keratinocytes (HaCaT cells) (Edlund et al., 2005).

Smad7 also blocks TGFβ induced anti-proliferative effects. In this case, Smad7 represses activation of Akt/PKB but enhances phosphorylation of c-Jun by TGFβ. Consequently, down-regulation of c-Myc, CDK4, and CyclinD1 and up-regulation

of $p21^{Cip-1}$ in response to TGFβ are reversed by ectopic expression of Smad7 (Halder et al., 2005). Similarly, in B-lymphocytes, the CD40 (TNF receptor superfamily member-5) signalling mediated activation of NF-κB up-regulates Smad7 expression and reverses TGFβ inducing anti-proliferation and apoptosis (Patil et al., 2000).

Thus, Smad7 either enhances or attenuates apoptosis in a context dependent manner. However, whether Smad7 induced apoptosis depends on TGFβ signalling or not needs to be elucidated. In some cases, Smad7 seems to induce apoptosis independent of TGFβ signalling (Davoodpour and Landstrom, 2005; Mazars et al., 2001).

5.1. Role of TGFβ signalling in myogenesis

It is well established that BMP signalling plays an important role for somite cell lineage specification. Therefore, Smad1 activation is a key step to regulate cell fate determination. The balance between the BMP signalling (SXS motif phosphorylation) and FGF/MAPK signalling (linker region phosphorylation) determine the Smad1 (Fuentealba et al., 2007; Kretzschmar et al., 1997a; Massague, 2003), or Smad 1/5/8 activity (Retting et al., 2009). The BMP4 signalling is antagonized by FGF10 in lung morphogenesis (Weaver et al., 2000), by FGF8 in tooth development (Neubuser et al., 1997), by FGF2 in cranial suture closure (Warren et al., 2003), by FGF4 in limb bud formation (Niswander and Martin, 1993a, b, c), and by FGF8 in retinoic acid inducing mouse embryonic carcinoma P19 cells differentiation into neuroectodermal cell lineages (Wang et al., 2006).

Smad2/3 activation by TGFβ is antagonized by the hyperactive Ras/MAPK signalling in cancer cells (Kretzschmar et al., 1999; Siegel and Massague, 2003). I-Smads also play an important role to regulate BMP signalling. BMPs and Smad6 and Smad7, are dynamically expressed in developing chick embryo limbs (Vargesson and Laufer, 2009). The BMP signalling represses myogenesis by up-regulation of Smad6. Smad6 brings Smurf1 to the T Box family-6 protein (Tbx6), which interacts with MH2 domain of Smad6 and Tbx6 is down-regulated by ubiquitin mediated-degradation. Since Tbx6 is a transactivator of the *myf5* gene, Myf5 expression, which plays a critical role for myogenic lineage determination, is inhibited by BMP (Li et al., 2006a; Yabe et al., 2006; Zhong et al., 2009). In C2C12, Smurf1 is required for myotube formation. Ectopic expression of Smurf1 reduces protein levels of Smad5 but surprisingly not Smad1, -2, -3, -7, and TGFβ receptors, and Smurf1 only interferes with osteoblast conversion by BMP2 but not inhibition of muscle differentiation by TGFβ. Therefore, Smurf1 is capable of blocking the BMP but not TGFβ signalling in C2C12 MBs (Ying et al., 2003).

5.1.1. Negative regulator of skeletal muscle differentiation by TGFβ

Although one study showed that in a mitogen-rich environment, TGFβ enhances muscle differentiation (Zentella and Massague, 1992), TGFβ is a well established inhibitor of muscle differentiation. TGFβ3 is expressed and secreted in skeletal muscle in embryos and adult and also in C2C12 MBs. Exogenously added TGFβ3 inhibits the MB fusion to form MTs (Lafyatis et al., 1991). The TGFβ type I and –II receptors are expressed in L6 rat MBs, and the TGFβ signalling is initiated by the

ligand/receptor complex formation (Segarini et al., 1992). In damaged skeletal muscle, prior to the inflammation reaction, TGFβ2 is produced by damaged necrotic muscle fibers, and then infiltrated macrophages generates TGFβ3-rich environment in the damaged area. Muscle regeneration is delayed until clearance of the macrophages from the necrotic fiber. Newly formed myotubes and activated satellite cells shows strong TGFβ2 immuno-reactivity (McLennan and Koishi, 1997). TGFβ inhibits muscle differentiation by down-regulation of Insulin-like growth factor-binding protein-5 (IGFBP5), and this down-regulation is mediated by JNK activation by TGFβ. Ectopic expression of a dominant negative form of MKK4, but not Smad7, reverses IGFBP5 down-regulation by TGFβ (Rousse et al., 2001). Therefore, TGFβ is an important regulator of skeletal muscle regeneration *in vivo*.

5.1.2. TGFβ activated signalling

Exogenously added TGFβ reversibly inhibits muscle differentiation *in vitro*, and it associates with strong induction of extracellular matrix type I collagen and fibronectin. It causes the dense multilayer cells on the cell culture plate (Massague et al., 1986). FGF also inhibits muscle differentiation (Spizz et al., 1987). TGFβ and bFGF up-regulates cyclin D1 expression, and ectopic expression of cyclin D1 inhibits muscle differentiation of C2C12 MBs (Rao and Kohtz, 1995). Both bFGF and TGFβ also up-regulate the fibroblast intermediate conductance calcium-activated potassium channel (FIK), a positive regulator of cell proliferation in a MEK/ERK pathway dependent manner. Both bFGF and TGFβ inhibit muscle

differentiation of 10T1/2-MRF4 cells (C3H10T1/2 stably expressing MRF4) depending on the activation of the Ras/ MEK/ERK kinase cascade and its down-stream target, FIK up-regulation (Pena et al., 2000). TGFβ also down-regulates the *igfbp5* gene expression independent of R-Smads, but depends on the activation of JNK (Rousse et al., 2001). TGFβ negatively targets the *myog* gene induction and up-regulates the *junB gene* in L6E9 rat MBs. Interestingly, ectopic expression of MyoG could not reverse TGFβ's inhibitory effect (Heino and Massague, 1990). Therefore, TGFβ inhibits muscle differentiation at the down-stream of the *myog* gene induction. Reduced expression of Decorin, a member of the small leucine-rich proteoglycan family, and/or lipoprotein-receptor related protein (LRP1) desensitizes the inhibitory effect of TGFβ (Cabello-Verrugio and Brandan, 2007; Riquelme et al., 2001). β-glycan is a membrane-anchored proteoglycan, which works as a co-receptor for TGFβ and bFGF through its glycosaminoglycan chains. β-glycan expression is up-regulated during C2C12 differentiation, and MyoD but not MyoG is responsible for this induction. TGFβ inhibits up-regulation of *β-glycan* gene (Lopez-Casillas et al., 2003). However, whether this down-regulation of *β-glycan* gene is responsible for the inhibitory effect of TGFβ on skeletal muscle differentiation was not addressed.

5.1.3. Role of SnoN and c-Ski

SnoN and c-Ski work as co-repressors of R-Smads (He et al., 2003), and c-Ski enhances skeletal muscle differentiation (Ichikawa et al., 1997). However, c-Ski's enhancement effect on myogenesis may not be due to inhibition of TGFβ R-Smad

'canonical' pathway. SnoN and c-Ski are targeted by sumolylation. However, the SUMO-modified SnoN and c-Ski and un-sumolylatable SnoN and c-Ski represses the R-Smads activity in the same extent, but the sumolylated SnoN and c-Ski markedly enhance skeletal muscle differentiation without affecting their protein levels (Wrighton et al., 2007). According to a recent report, c-Ski is able to displace HDAC from MyoD and de-represses MyoD's transcriptional properties independent of the TGFβ R-Smad pathway (Kobayashi et al., 2007). Therefore, regulator of c-Ski other than the TGFβ signalling might be important for muscle differentiation.

5.1.4. Role of $p57^{Kip-2}$

TGFβ causes proteolysis of cyclin-dependent kinase inhibitor 1C ($p57^{Kip-2}$) and has a unique role in embryo development (Westbury et al., 2001) and muscle differentiation (Reynaud et al., 2000; Reynaud et al., 1999). The degradation of $p57^{Kip-2}$ is enhanced by the R-Smad mediated up-regulation of the ubiquitin-proteasome activity. TGFβ induces proliferation of osteoblasts by the degradation of this CKI, and ectopic expression of Smad7 blocks this pro-proliferative effect of TGFβ in osteoblasts (Nishimori et al., 2001; Urano et al., 1999). However, whether TGFβ targets $p57^{Kip-2}$ for the inhibition of myogenesis has not been addressed.

5.1.5. Role of MEF2C

One group reported that an activated Smad3 by TGFβ interferes with MEF2C transactivation properties by the interaction with the MADS domain of MEF2C and inhibits myogenesis. This interaction interferes with the recruitment of a co-

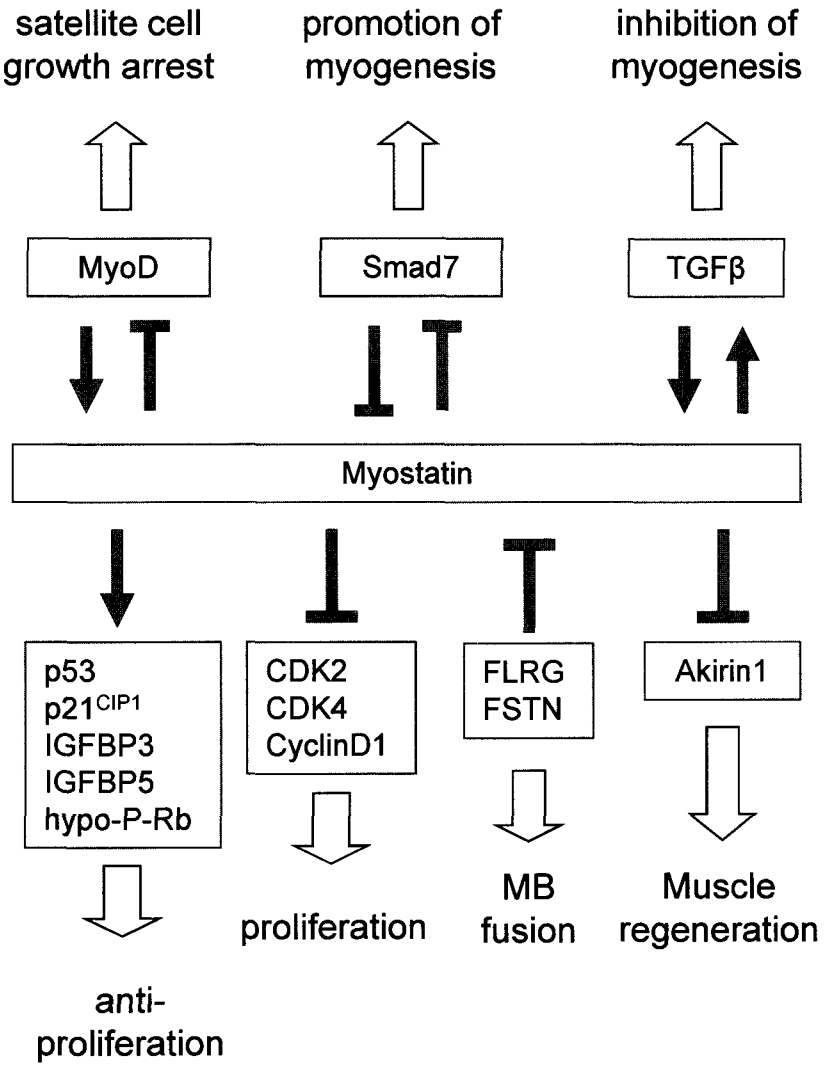


Figure 30. **Role of Myostatin in myogenesis**

activator GRIP1 to the MEF2C (Liu et al., 2004). However, the *mef2C* gene expression is up-regulated later than MRF is in muscle differentiation (Martin et al., 1993). Therefore, it is questionable whether inhibition of MEF2C transcriptional properties by Smad3 is responsible for inhibition of myogenesis by TGFβ. Finally, our group documented that an inhibitor of the TGFβ/R-Smad 'canonical' pathway, Smad7, potently inhibits the R-Smad activity in response to TGFβ, but ectopic expression Smad7 could not reverse the inhibitory effect of TGFβ on muscle differentiation (Kollias et al., 2006). Thus, R-Smad activation may not be required for the inhibition of myogenesis by TGFβ.

6.1. Role of Myostatin in myogenesis

Myostatin/Growth differentiation factor-8 (MSTN/GDF8), a member of the TGFβ family, is a potent physiological reversible negative regulator of skeletal muscle growth. Naturally occurring loss of function mutations of the *mstn* gene cause greatly increased skeletal muscle mass (Grobet et al., 1997; McPherron et al., 1997; McPherron and Lee, 1997) (figure 30).

6.1.1. Regulation of myostatin (mstn) gene expression

MyoD positively regulates the *mstn* gene transcription through multiple E-boxes in the promoter. Since MSTN has anti-proliferative effect, MyoD may utilize MSTN to withdraw from the cell cycle (Spiller et al., 2002). Interestingly, ectopic expression of Smad7 reduced the promoter activity of the *mstn* gene, and MSTN down-regulates its own promoter activity though up-regulation of Smad7 (Forbes et al., 2006). Our group documented that ectopic expression of Smad7 reverses

Myostatin's inhibitory effect on skeletal muscle differentiation (Kollias et al., 2006). In addition, in C2C12 MBs, MSTN up-regulates TGFβ production, and TGFβ also stimulates the *mstn* gene expression, and Decorin induces Follistatin and antagonises MSTN action (Zhu et al., 2007) (figure 30).

6.1.2. Anti-proliferative effect of MSTN

Myostatin inhibits proliferation of C2C12 MBs through up-regulation of $p21^{Cip-1}$ and down-regulation of CDK2 (Thomas et al., 2000). Ectopic expression of MSTN up-regulates p53, $p21^{Cip-1}$, IGFBP3, and IGFBP5, and hypophosphorylated Rb (Joulia et al., 2003; Kamanga-Sollo et al., 2005). MSTN also down-regulates nuclear cyclinD1 by both degradation and nuclear export dependent on GSK3β activity. As a consequence, CDK4 activity is reduced by MSTN (figure 30). IGF1 and PI3K/Akt signalling reverse the cyclinD1 down-regulation and anti-proliferative effect by MSTN. If IGF1 signalling is inhibited, upon MSTN stimulation, C2C12 cells are eliminated by apoptosis. Furthermore, IGF1 up-regulates the *mstn* gene expression depending on the PI3K activation (Yang et al., 2007). MSTN also inhibits translation through down-regulation of Akt/mammalian target of rapamycin (mTOR) pathway (Amirouche et al., 2009).

6.1.3. MAPK activation by MSTN

Myostatin activates the Ras/MEK/ERK kinase cascade in C2C12 cells, and a MEK specific inhibitor, PD98059 reverses the MSTN's inhibitory effect on muscle differentiation. Therefore, MSTN inhibits myogenesis by activation of the MAPK cascade (Yang et al., 2006). MSTN also activates JNK in C2C12, and similarly,

JNK inhibitor, SP600125, reverses $p21^{Cip-1}$ up-regulation and MRF down-regulation by MSTN (Huang et al., 2007). MSTN activates p38MAPK through TAK1, and ATF2 is phosphorylated in response to MSTN (Philip et al., 2005).

6.1.4. Inhibition of satellite cell activation by MSTN

Myostatin up-regulates $p21^{Cip-1}$ and down-regulates CDK2 in the satellite cells, and conversely, the *mstn*-/- satellite cells shows enhanced proliferation and consequently higher numbers of satellite cells. Therefore, MSTN inhibits the 'self-renewal' of satellite cells (McCroskery et al., 2003). In chick muscle development, MSTN down-regulates Pax3 and MyoD, and this inhibition can be reversed by Follistatin which antagonises MSTN activity by direct protein-protein interaction (Amthor et al., 2004). In addition, MSTN down-regulates Myf5 and MyoG but no effect on Pax7. This inhibitory effect is reversible, and upon removal MSTN, the stimulated satellite cells up-regulate Pax3 and MyoD and proliferate normally (Amthor et al., 2006).

6.1.5. Inhibition of muscle regeneration by MSTN

The product of the follistatin-related gene (*flrg*) associates with MSTN and restricts MSTN activity in the circulation (Hill et al., 2002). Since Follistatin (FSTN) is involved in the MB fusion independent of IGF1 or IL-4, MSTN inhibition also mimics the FSTN effect. Interestingly, a HDAC inhibitor, trichostatin A, increases FSTN expression and enhances the expression of regeneration marker genes (Iezzi et al., 2004). In the *mstn* -/- adult mouse, muscle regeneration is accelerated, and the satellite cell migration and the recruitment of the macrophages into the damaged

sites and their clearance is enhanced, in addition, fibrosis is reduced (McCroskery et al., 2005).

MSTN also enhances interaction between MyoD and Smad3 which interferes with MyoD's transactivation properties. However, ectopic expression of DN-Smad3 partially reversed this effect but ectopic expression of MyoD had no effect on the inhibitory effect of MSTN (Langley et al., 2002). Akirin1 (Mighty) is a potential signal transducer in muscle regeneration. Akirin1 is up-regulated in the activated proliferating satellite cell. Macrophages also express Akirin1, and the increase in the chemotaxis of the macrophages and MBs can be observed by increased expression of Akrin1. Importantly, Akirin1 is a negative target of MSTN (Salerno et al., 2009) (figure 30). Function of Akirin1 in muscle regeneration needs to be characterized.

6.1.6. Pathology by hyperactivation of Myostatin

A DN-mutation of muscle specific Caveolin, Caveolin-3, causes autosomal dominant limb-girdle muscular dystrophy 1C. The loss of function of Caveolin-3 hyper-activates MSTN and causes muscle atrophy. The wild type Caleolin-3 antagonises the MSTN signalling through inactivation of Smad2 by decreasing its phosphorylation (Ohsawa et al., 2006). The blockade of MSTN signalling may prevent depletion of the satellite cells in the dystrophic muscles (Bogdanovich et al., 2002). Administration of a soluble form of the activin type IIB receptor, which binds MSTN and blocks binding to the cell surface receptor, increases muscle mass (Lee et al., 2005b).

6.1.7. Adipocyte

BMP2 and -7 enhance adipogenesis, and MSTN antagonises this adipogenic effect of the BMPs by competing out the BMPs from binding to the receptors (Rebbapragada et al., 2003). However, MSTN by itself enhances differentiation of pluripotent C3H10T1/2 fibroblasts into immature adipocytes. It mimics the effect of glucocorticoid/dexamethasone. These immature adipocytes exhibit increased insulin sensitivity and glucose oxidation and resists to diet-induced obesity (Feldman et al., 2006; Guo et al., 2009). Conversely, glucocorticoid-induced muscle atrophy can be 'rescued' by the *mstn* -/- mouse (Gilson et al., 2007)

7.1. Role of Smad7 in myogenesis

Smad7 inhibits the ActRIB/ALK4 activation by Activin and BMP and blocks activation of dorsal mesoderm genes (Casellas and Brivanlou, 1998). Consequently, Smad7 up-regulates neural makers and inhibits mesoderm formation (Bhushan et al., 1998). Osteoblast differentiation induced by the BMP6/R-Smad pathway is inhibited by ectopic expression of Smad7 and Smad6 (Fujii et al., 1999). Furthermore, Smad7, but not Smad6, interferes with the TGFβ mediated inhibition of both lung branching morphogenesis and cyto-differentiation (Zhao et al., 2000). A large number of studies documented that the TGFβ signalling is a key regulatory pathway for myogenesis, and it has been speculated that Smad7 is an important molecule for biological events regulated by the TGFβ signalling pathway. However, a physiological role of Smad7 has not been clearly defined yet because most studies focus on Smad7 as an inhibitor of the TGFβ 'canonical' signalling pathway.

Our group documented that Smad7 is required for skeletal muscle differentiation by utilizing siRNA technology to reduce endogenous expression of Smad7 (Kollias et al., 2006). With the reduced levels of Smad7, up-regulation of MyoG, MyoD, MEF2A, and MyHC that are up-regulated in the normal course of muscle differentiation, was attenuated without affecting on the Smad2/3/4 protein levels and Smad2/3 phosphorylation. Since the reduction of Smad7 activates Smad3 dependent reporter gene activity, endogenous Smad7 works as an inhibitor of TGFβ 'canonical' pathway. Therefore, one can still argue that reduced levels of Smad7 causes the hyperactive TGFβ 'canonical' signalling and inhibits myogenesis. However, our gain of function approach indicated that TGFβ does not inhibit myogenesis by the activation of R-Smads (Kollias et al., 2006). Ectopically expressed Smad7 potently inhibited the R-Smad dependent reporter gene activity in the presence of TGFβ. However, inhibitory effect of TGFβ but not MSTN was not reversed by silencing the TGFβ 'canonical' pathway by ectopic expression of Smad7. These observations imply that TGFβ and MSTN inhibit myogenesis in different manners, and activation of R-Smad by TGFβ is not sufficient to explain the mechanism by which TGFβ inhibits myogenesis. In addition, ectopic expression of Smad7 not only reverses but greatly enhances myotube formation in the presence of MSTN suggesting that Smad7 promotes skeletal muscle differentiation other than inhibiting the TGFβ/Myostatin 'canonical' pathway. In agreement with this hypothesis, Smad7 physically interacts with MyoD which is a nuclear protein, and enhances MyoD's transactivation properties (Kollias et al., 2006). Therefore, Smad7 may also promote myogenesis as a positive co-activator of MyoD, and it is

independent of the inhibitory role of Smad7 in the TGFβ signalling at receptor level. Finally, we also found that the *smad7* gene is a MyoD target in muscle cells (Kollias et al., 2006). Since the *smad7* gene is up-regulated in response to TGFβ, Smad7 was characterized as part of a negative feedback loop of TGFβ 'canonical' pathway (Nakao et al., 1997). However, in C2C12 MBs, mutation of the SBE, which mediates TGFβ/R-Smad inducibility, had not a clear effect on the *smad7* promoter activity; in contrast, the E-box mutation caused greatly reduced the *smad7* promoter activity. In addition, ectopic expression of MyoD potently activated the *smad7* promoter-reporter gene activity (Kollias et al., 2006) (figure 31). Therefore, Smad7 is an integrated part of the myogenic gene expression program.

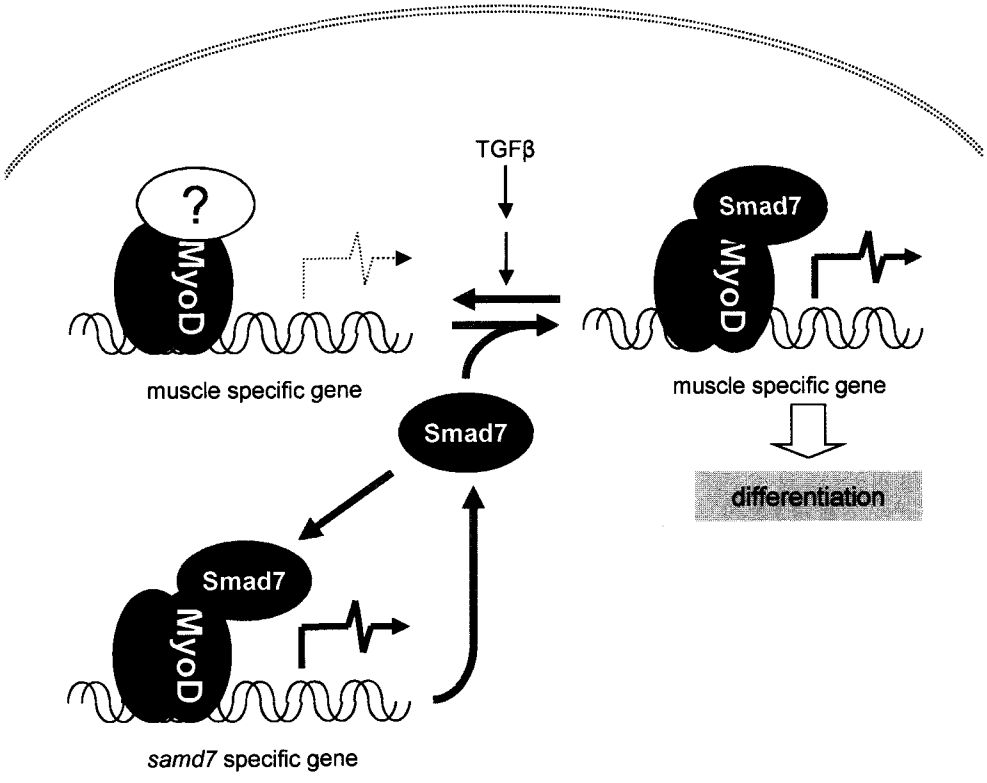


Figure 31. **Role of Smad7 in muscle differentiation**

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Chapter II; Statement of Purpose

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In MBs, MyoD and Myf5 are already expressed, but these MRF's transactivation properties are repressed until stimulated by extracellular cues for muscle differentiation or regeneration. Once MyoG is induced by MyoD in co-operation with MEF2, the MBs terminally differentiate into MTs. Therefore, the regulation of MyoD's transactivation properties is a key event to determine MB cell fate, and CT-1 and TGFβ are candidate molecules to control this event. The primary goal of these studies was to characterize the role of cytokines such as CT-1 and TGFβ in myogenic cells.

Cardiotrophin-1 maintains the undifferentiated state of skeletal myoblasts (Chapter III)

Although CT-1 is highly expressed in skeletal muscle during embryo development and in adulthood, the role of CT-1 in skeletal muscle had not been well characterised. Previous studies documented that in cardiomyocytes, CT-1 activates MEK/ERK/AP-1 and JAK/STAT signalling pathways, and both AP-1 and STAT are inhibitory molecules for skeletal myogenesis. Therefore, we sought to characterize how CT-1 affects skeletal muscle differentiation at the molecular level.

Nuclear Smad7 enhances skeletal muscle differentiation (Chapter IV)

Previous work from our group documented that Smad7 promotes skeletal muscle differentiation. Interestingly, we found that Smad7 physically interacts with nuclear transcription factor MyoD and enhances its transactivation properties. In addition, although Smad7 potently inhibits TGFβ 'canonical' pathway, ectopic expression of

Smad7 could not reverse the inhibitory effect of TGFβ on myogenesis. Since most of studies focus on the inhibitory aspect of Smad7 at the receptor level even though Smad7 localized mainly in the nucleus, we, therefore, sought to characterize the nuclear role of Smad7.

Maintenance of the undifferentiated state in skeletal myoblasts by TGFβ is Smad independent and requires MEK activation (Chapter V)

Since our previous study suggested that TGFβ might inhibit skeletal muscle differentiation independent of activation of R-Smad and other studies documented that TGFβ activates the MAPK pathway in different systems, we hypothesized that TGFβ inhibits myogenesis by activation of MEK independent of activation of R-Smad.

Chapter III; Cardiotrophin-1 maintains the undifferentiated state in skeletal myoblasts

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Experimental design and drafting manuscript
Tetsuaki Miyake and Dr. John C McDermott

Conducting experiments
Tetsuaki Miyake; (figure 1B, 3, 4A, 4B, 4D, 4E, 5A, 5B, 5C, 5D, 5E, 6A, 6B, 6C, 6D, and Supplemental figure 1 and 2)

Nezeka S Alli and Arif Aziz; (figure 1A, 1C, 1D, 2, and 4C)

Jennifer Knudson, Pasan Fernando, and Dr.Lynn A. Megeney; (figure 7A, 7B, 7C, 7D, and 7E)

Cardiotrophin-1 maintains the undifferentiated state in skeletal myoblasts

Tetsuaki Miyake*, Nezeka S. Alli*, Arif Aziz*, Jennifer Knudson¤, Pasan Fernando¤, Lynn A. Megeney¤, and John C. McDermott*

*Department of Biology, York University, 4700 Keele street, Toronto, Ontario, M3J 1P3 Canada. ¤The Sprot Center for Stem Cell Research, Regenerative Medicine Program, Ottawa Health Research Institute, 501 Smyth Road, Ottawa, Ontario, K1H 8L6, Canada.

correspondence to: John C.McDermott, Department of Biology, 327 Farquharson, LSB, York University, 4700 Keele St., Toronto, Ontario, Canada. M3J 1P3
Fax:(416)-736-5698, E-mail: jmcderm@yorku.ca

Running title: CT-1 inhibits skeletal myogenesis.

Abstract

Skeletal myogenesis is potently regulated by the extracellular milieu of growth factors and cytokines. We observed that Cardiotrophin-1 (CT-1), a member of the Interleukin-6 (IL-6) family of cytokines, is a potent regulator of skeletal muscle differentiation. The normal up-regulation of myogenic marker genes, Myosin Heavy Chain (MyHC), Myogenic Regulatory Factors (MRFs), and Myocyte Enhancer Factor 2s (MEF2s) were inhibited by CT-1 treatment. CT-1 also represses Myogenin (MyoG) promoter activation. CT-1 activated two signaling pathways: Signal Transducer and Activator of Transcription-3 (STAT3), and Mitogen-Activated Protein Kinase Kinase (MEK), a component of the Extracellular Regulated MAP Kinase (ERK) pathway. In view of the known connection between CT-1 and STAT3 activation, we surprisingly found that pharmacological blockade of STAT3 activity had no effect on the inhibition of myogenesis by CT-1 suggesting that STAT3 signaling is dispensable for

myogenic repression. Conversely, MEK inhibition potently reversed the inhibition of myotube formation and attenuated the repression of MRF transcriptional activity mediated by CT-1. Taken together, these data indicate that CT-1 represses skeletal myogenesis through interference with MRF activity by activation of MEK/ERK signaling. In agreement with these *in vitro* observations, exogenous systemic expression of CT-1 mediated by adenoviral vector delivery increased the number of myonuclei in normal post-natal mouse skeletal muscle and also delayed skeletal muscle regeneration induced by cardiotoxin (CTX) injection. The expression pattern of CT-1 in embryonic and post-natal skeletal muscle and *in vivo* effects of CT-1 on myogenesis implicate CT-1 in the maintenance of the undifferentiated state in muscle progenitor cells.

Introduction

Terminal differentiation of skeletal myogenic cells, termed myogenesis, consists of a series of well characterized highly regulated steps that has become a paradigm for lineage acquisition and cellular differentiation. Initially, pluripotent mesodermal stem cells commit to become myogenic precursor cells. Commitment to the myogenic lineage then results in the binary state of either maintenance of proliferative potential and pluripotency, or, on appropriate cues, withdrawal from the cell-cycle, activation of a battery of structural, contractile and metabolic genes constituting the differentiation programme and ultimately formation of multi-nucleated myotubes (Perry and Rudnick, 2000). The field of myogenesis has benefited from the use of well established *in vitro* cell-culture systems which faithfully recapitulate the *in vivo*

differentiation programme. During myogenesis, a group of basic helix-loop-helix transcription factors, Myogenic Differentiation-1 (MyoD), Myogenic Factor-5 (Myf5), Myogenin (MyoG), and Myogenic Regulatory Factor-4 (MRF4), collectively termed the Myogenic Regulatory Factors (MRFs), play essential roles in differentiation (Kassar-Duchossoy et al., 2004; Pownall et al., 2002; Rudnicki and Jaenisch, 1995). Most promoter-enhancer regions of muscle specific genes contain the cognate binding site, E-box (CANNTG), for the MRFs, and the E-box is often essential for the induction of these genes during differentiation (Tapscott, 2005; Walsh and Gualberto, 1992). For example, early and late muscle specific genes, MyoG and muscle specific Myosin Heavy Chain (MyHC) respectively, are transcriptionally regulated by MyoD and other MRFs through E-boxes in their proximal promoter regions (Penn et al., 2004; Rudnicki and Jaenisch, 1995). The molecular and genetic requirement for the MRFs during myogenesis has been confirmed in many studies both *in vitro* and *in vivo* (Edmondson and Olson, 1989; Kassar-Duchossoy et al., 2004; Myer et al., 2001). The MRFs also co-operate with another class of myogenic transcription factors, comprised of the Myocyte Enhancer Factor two family (MEF2) (Naya and Olson, 1999; Olson et al., 1995). MEF2 genes are taxonomically part of the MADS-box gene super-family that encode DNA binding proteins involved in yeast mating type decisions (MCM1), plant development (Agamous and Deficiens), and serum responsivity of mammalian cells (Serum Response Factor: SRF) (Jarvis et al., 1989; Norman et al., 1988; Sommer et al., 1990; Yanofsky et al., 1990).

As well as the detailed knowledge of core transcriptional regulatory circuits mediated by myogenic transcription factors and their accessory factors, much work has

contributed to the identification of a number of growth factor and cytokine mediated signaling pathways that positively and negatively impact myogenesis (Engert et al., 1996; Olwin and Hauschka, 1988; Ridgeway et al., 2000; Templeton and Hauschka, 1992). In some cases, these pathways regulate the decision to differentiate or not, a critical regulatory point since differentiation in muscle is terminal and absolutely required for viability of all metazoan life. Moreover, negative regulation of differentiation is equally important since it underpins the maintenance of the proliferative state and pluripotency.

A number of growth factors and cytokines, such as Insulin like Growth Factors (IGFs), insulin, Transforming Growth Factor-β (TGFß), Fibroblast Growth Factor (FGF), and Epidermal Growth Factor (EGF), that influence myogenesis have been identified (Allen and Boxhorn, 1987; Engert et al., 1996; Olwin and Hauschka, 1988; Templeton and Hauschka, 1992), however, a detailed understanding of their corresponding signal transduction pathways and transcriptional network targets is still rudimentary. One group of cellular signaling cascades that are known to affect myogenesis in a complex manner are the MAPK pathways. For example, p38 MAPK, a member of one of the MAPK pathways, directly phosphorylates and activates E47, which forms a productive dimer with MyoD (Lluis et al., 2005; Lluis et al., 2006). P38MAPK also regulates MEF2 (Cox et al., 2003; Zetser et al., 1999) transcription factors as well as being involved in the recruitment of ATP dependent chromatin remodelling factors to myogenic loci (de la Serna et al., 2005; McKinsey et al., 2002; Serra et al., 2007). Conversely the ERK-MAPK cascade plays a bi-phasic role in myogenic cells, being inhibitory in the initial phases of the differentiation program

while being required for later stage events, such as cell fusion (Bennett and Tonks, 1997).

CT-1 is a member of the IL-6 family, which is comprised of IL-11, Leukemia inhibitory factor (LIF), Ciliary Neurotrophic Factor (CNTF), and Oncostatin M (OSM). These cytokines are structurally related and form a variety of oligomeric ligand – receptor complexes. IL-6 and IL-11 form a complex with a homodimer of the Glycoprotein-130 (Gp130) receptor or heterodimers of gp130 and leukaemia inhibitory factor receptor-β (LIFRß). Gp130/LIFRβ also recognises LIF, CT-1, CNTF, OSM, and Cardiotrophin-Like Cytokine (CLC). OSM binds to the Gp130 and Oncostatin M receptor (OMR). Upon formation of the requisite complex with the respective cytokine, the preponderant view is that the oligomeric receptor complex transduces its signal through the Janus kinase (JAK)-STAT signaling pathway (Heinrich et al., 2003).

CT-1 was originally identified in conditioned medium from embryoid bodies (Pennica et al., 1995). In developing embryos, CT-1 is expressed in heart, skeletal muscle, liver and dorsal root ganglia (Sheng et al., 1996). In adults, human CT-1 mRNA is detected in the heart, skeletal muscle, ovary, colon, prostate and testis, and in fetal kidney and lung (Pennica et al., 1996). The functions of CT-1 in the cardiovascular system have been extensively researched. Patients with ischemic and valvular heart disease have elevated levels of CT-1 in their sera (Freed et al., 2003). Further study of the role of CT-1 in the heart indicated that it has a cardio-protective role by reducing apoptosis (Brar et al., 2001; Sheng et al., 1996) and may be involved in regeneration of cardiac muscle after infarction (Freed et al., 2005). Exogenously

administered CT-1 also induces cardiac hypertrophy *in vitro* (Sheng et al., 1996). While the modulation of cardiomyocyte phenotype by CT-1 has been well documented, the underlying signaling pathways are still unclear and the role of CT-1 in skeletal muscle has not, thus far, been characterized.

In this report, we demonstrate that CT-1 is a potent inhibitor of skeletal muscle differentiation. In C2C12 cells, CT-1 represses molecular markers of muscle differentiation and phenotypic myogenesis. Also, the transcriptional networks involved in the induction of key myogenic genes such as the MyoG and MCK genes are suppressed by CT-1 signaling. Surprisingly, small chemical inhibitors of MEK, PD98059 and U0126, reversed these repressive effects on skeletal myogenesis by CT-1 whereas inhibition of STAT3 activation was without effect. Collectively, these data show that CT-1 interferes with the transcriptional network required for muscle differentiation through the activation of the MEK-MAPK signaling module. Furthermore, *in vivo*, adenovirus mediated expression of CT-1 increases satellite cell number and delays regeneration of damaged muscle by cardiotoxin (CTX) injection. These observations indicate that CT-1 represses myogenesis and serves to maintain myogenic progenitors in their proliferative, multipotent state *in vitro* and *in vivo*.

Experimental procedures

Plasmids MRF expression plasmids were constructed in pEMSV as described elsewhere (Davis et al., 1987). An activated (ΔN3 S218D/S222E) human MEK1 expression construct was a kind gift from A. Natalie (Mansour et al., 1994). The reporter construct pMCK-eGFP was a gift from A. Ferrer-Martinez (Universitat de Barcelona, Spain). Transcription reporter constructs, pMCK-luc (Donoviel et al., 1996), pCMV-β-Galactosidase were described elsewhere (Kollias et al., 2006). The myogenin promoter region was excised from pMyoG-luc by *SacI /Bgl II* digestion. The resultant 1152bp fragment was inserted at the SacI */Bgl II* sites of pGL4-10 vector (Promega). The dsRed2-N1 expression construct was purchased from Clontech Laboratories.

Antibodies The primary antibodies used in this study were obtained from Santa Cruz Biotechnology; MyoD (C-20), Myf5 (C-20), Actin (I-19), and ERK1 (C-16), from BD Biosciences; MEF2D (610775), from Cell Signaling Technology; Stat3 (9132), Phospho-Stat3 (Tyr705) (58E12; 9135), Phospho-Stat3 (Ser727) (6E4; 9136), MEK1/2 (9122), Phospho-MEK1/2 (Ser217/221) (9121), Phospho-p44/42 MAPK (Thr202/Tyr204) (E10; 9106), from Developmental Studies Hybridoma Bank; Myogenin (F5D), and MyHC (MF20), from DakoCytomation; MyoD1 (clone:5.8A; M3512). Polyclonal antibody for MEF2A was prepared as previously described (Cox et al., 2003). Normal mouse (sc-2025) IgG was from Santa Cruz Biotechnology.

Cell Culture C2C12 myoblasts were obtained from American Type Culture Collection (CLR-1772) and cultured in growth medium (GM) consisting of 10% Fetal bovine serum (FBS) (HyClone) in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 1% penicillin-streptomycin (Gibco) at 37 °C and 5% CO_2. Myotube formation was induced by replacing GM with differentiation medium (DM) which consisted of 2% horse serum (Atlanta Biologicals) in DMEM supplemented with 1% penicillin-streptomycin. For CT-1 treatment, recombinant mouse CT-1 (R&D system; 438-CT) was resuspended with solvent (4 mM HCl, 0.1% bovine serum albumin (BSA)) and supplemented into the media. For myotube formation assays, DM with CT-1 (10ng/ml) was replenished every 2 days. Inhibitors (PD98059 (Cell Signaling Technology; 9900), U0126 (Cell Signaling Technology; 9903), and P6 (2-(1,1-Dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one; Pyridone 6) (Calbiochem; 420097)) were resuspended with DMSO and added into the cell culture media for 30 minutes prior to adding CT-1.

Sacromeric Myosin Heavy Chain Detection

C2C12 cells were washed with Phosphate buffered saline (PBS) (pH7.4) and fixed with 90% methanol at -20 °C for 10 min. After fixation, the cells were incubated in 5% milk in PBS for 30 min at 37 °C for blocking. Cells were incubated at room temperature with MF-20 (primary antibody) diluted in blocking buffer (5% milk PBS) for 1 hour. After incubation, the cells were washed three times with PBS and incubated for 60 min at room temperature with an Horseradish peroxidase (HRP)-

conjugated α-mouse secondary antibody. The cells were again washed three times with PBS and incubated in developer (0.6 mg/ml DAB, 0.1 % H_2O_2 in PBS) to detect MyHC by immunocytochemistry. The nuclei were counter-stained with haematoxylin. Images were recorded with a microscope (Axiovert 35; Carl Zeiss MicroImaging) with either 4X NA 0.10 or 10X NA 0.25 Achrostigmat objective lenses with a digital camera (Canon, EOS D60).

Proliferation assay After 72hrs in DM and in the presence of CT-1 (10ng/ml) (or solvent), cells were incubated with 100μM of BrdU (Sigma) for 1 hr at 37°C. Cells were washed with cold 1x PBS then fixed with 70% ethanol for 1hr at 4°C. The cells were then washed with 1x PBS and incubated with 2N HCl for 1 h at 37°C to denature the DNA. The cells were blocked in 10% goat serum (Sigma) diluted in 1x PBS for 2hrs at room temperature with shaking and then incubated with BrdU primary antibody (G3G4: Developmental hybridoma bank, Iowa) diluted in 1.5% goat serum (Sigma) for 1.5hr at room temperature with shaking. Cells were washed with 1x PBS-T (0.5% Tween20) and incubated with anti-mouse secondary antibody conjugated to FITC (Sigma) diluted in 1.5% goat serum (Sigma) for 2hrs at room temperature with shaking. Cells were washed with 1x PBS-T (0.5% Tween20).

Microscopy and Fluorescence Fluorescence and phase contrast pictures were obtained using an epifluoresence microscope (Axiovert 35; Carl Zeiss MicroImaging), with appropriate phase and filter settings, and either 4X NA 0.10 or 10X NA 0.25

Achrostigmat objective lenses. Images were recorded with a digital camera (Canon, EOS D60).

Western blotting analysis Total cellular protein extracts were prepared in NP-40 lysis buffer (0.1 % NP-40, 150 mM NaCl, 1mM EDTA, 50 mM Tris-HCl pH 8.0, 1mM sodium vanadate, 1mM PMSF, supplemented with a protease inhibitor cocktail (Sigma, P-8340)). Protein concentrations were determined by a standard Bradford assay (BioRad). Equivalent amounts of protein were resolved by SDS-PAGE gels, followed by electrophoretic transfer to an Immobilon-P membrane (Millipore) as directed by the manufacturer (Millipore). Blots were incubated with the indicated primary antibody in 5% milk in PBS or Tris buffered saline (TBS)-T (10mM Tris-HCl pH8.0, 150mM NaCl, 0.1% Tween-20) or 5% Bovine serum albumin (BSA) in TBS-T according to the manufacturer's protocol at 4 °C overnight with gentle agitation. After washing briefly, the blots were incubated with the appropriate HRP-conjugated secondary antibodies in 5% milk in PBS or TBS-T at room temperature according to the manufacturer's protocols (Santa Cruz Biotechnology, Cell Signaling Technology). After washed three times with 1XPBS or 1XTBS (depending on the primary antibody) at room temperature, the blots were treated with the Enhanced chemiluminescence reagent (Amersham) to detect immuno-reactive proteins. The blots were exposed to Biomax film (Kodak) for visual representation.

Transcription reporter gene assays C2C12 myoblasts were transfected by a standard calcium phosphate-DNA precipitation method with the indicated reporter

gene and expression constructs and pCMV-β-Galactosidase to monitor transfection efficiency. After transfection, the cells were washed with PBS and maintained in GM and then treated as indicated. Total cellular protein was extracted with luciferase lysis buffer (20mM Tris-HCl pH7.4, 0.1% Triton X-100). Luciferase and β-Galactosidase enzyme assays were performed according to the manufacturer's protocol (Promega). Luciferase activity was quantified using a luminometer (Berthold Lumat, 9501) and standardized according to the β-Galactosidase activity. Relative Luciferase units normalized for the β-Galactosidase activity (Relative Luciferase Unit; RLU) were determined and plotted as an average of triplicate determinations and error bars represent standard deviations of the triplicate values. Each experiment was repeated at least three times.

Semi-quantitative RT-PCR analysis Total RNA was extracted from cells with TRIzol (invitrogen) according to manufacture's protocol. cDNA was generated from the isolated total RNA (1μg) with SuperScript III (invitrogen) and oligo-dT $_{(16)}$ primer (Sigma) by the protocol provided by the manufacturer. To amplify a target transcript, a pair of primers was designed that flanked an intron based on the mouse gene sequences. The target transcripts were amplified by *taq* DNA polymerase (New England Biolab) with gene specific primers. An amplified DNA was separated in an agarose gel and visualized by ethidium bromide (EtBr) (Sigma) staining and UV exposure. Detailed information about the primers is in supplement.

Co-immunoprecipitation analysis An equal amount of total cellular protein (250μg) was diluted with NP-40 lysis buffer to a final concentration of 1 μg/μl. Protein complexes were immunoprecipitated with the indicated antibody and 25μl of protein G-Plus Sepharose beads (50% slurry) (Santa Cruz Biotechnology) by incubation at 4°C overnight on a rotating platform. The beads were washed with three changes of NETN wash buffer (0.1% NP-40, 150mM NaCl, 1mM EDTA, and 50 mM Tris-HCl pH 8.0). Beads were boiled in SDS sample buffer, and protein complexes were resolved by SDS-PAGE and immuno-blotted as described above.

CT-1 adenovirus The CT-1 adenovirus was previously described (Bordet et al., 1999). Briefly, full length murine CT-1 cDNA was isolated by PCR and the CT-1 reading frame was fused with a 60 base pair pre-Nerve Growth Factor (NGF) leader sequence to promote secretion of the CT-1 protein. The CT-1 cDNA was cloned in the Rous Sarcoma virus (RSV) vector (Bordet et al., 1999). A LacZ containing adenovirus (CTRL) was used as a control for all injection experiments. This adenovirus was kindly provided by Dr. Robin Park at the Ottawa Health Research Institute, Ottawa, Canada.

***In vivo* administration of CT-1: Muscle injury**

To test CT-1 in vivo, B6C3F1 mice were subjected to systemic delivery of the CT-1 adenovirus. Briefly, animals were anaesthetized with halothane. The injections were administered via intra-cardiac chamber delivery using a 29-gauge insulin needle (VWR) with 50μL of Ad-CT-1 at a concentration of 3.0 X 10^8 PFU/mL (n=3). A

control group of B6C3F1 mice were injected with 50μL Ad-CTRL at a similar concentration (n=3). In a separate group of animals, cardiotoxin was used to induce muscle injury immediately prior to AdCT-1 and Ad-CTRL injection (n=3 for each group). 25μl of 10μM cardiotoxin (Latoxan) was injected directly into the TA muscle using a 29G1/2 insulin syringe in halothane anaesthetized mice (Asakura et al., 2002). Post-recovery, mice were monitored closely for weight loss, dehydration and cardiac distress. All injections were administered by a trained animal care technician according to the standards of the Animal Care Committee at the University of Ottawa, Ottawa, Canada.

Immuno-histology At 7days post-injection, skeletal muscle was excised and rinsed in cold 1XPBS. The muscle was fixed in 4% PFA in PBS for 2days then embedded in paraffin, sectioned at 10μM and counterstained with haematoxylin and eosin to visualize the nuclei and cytoplasm. Sections were dehydrated in a graded ethanol series ending in CitriSolv (Fisher Scientific). For immuno-histological, sections were treated with antigen unmasking solution (Vector Labs), blocked with 5% BSA, incubated overnight at 4°C with a primary antibody, then incubated in donkey-anti-goat CY3 antibody (Chemicon) and finally counterstained with DAPI (Sigma). 5 fields of view per section and 5 sections per TA muscle were analysed. The micrographs were representative views.

Stem Cell/Progenitor Cell Isolation Side population (muscle progenitor cells) were collected as previously described (Hierlihy et al., 2002). Contra lateral TA

muscle was collected from Ad-CT-1 and Ad-CTRL mice and all visible connective tissue and blood vessels were removed by dissection. Muscle was digested in collagenase B (10mg/mL) (Roche) plus dispase II (2units/mL) (Roche) for and the resulting single ell suspensions were then stained with Hoechst dye 33342 (5ug/mL) (Sigma-Aldrich) at 37°C for 90 minutes. As an SP control, the drug verapamil (50μM) (Sigma-Aldrich) was added to an aliquot of cells simultaneously stained with Hoechst 33342. Cells were finally re-suspended in 500μL of Hanks Balanced Salt solution with 2% FBS and 10mM Hepes (HBSS+). The cells were filtered through a 50μM Cell Tric® (disposable filters made of monofil nylon material) (Partec GmbH) and remained on ice until FACS analysis (Hierlihy et al., 2002). Cell sorting was performed using a DakoCytomation MoFlo high-speed cell sorter (DakoCytomation) (Hierlihy et al., 2002). Forward and side scatter was measured at 488 nm (Spectraphysic Argon Laser). The Hoechst dye was excited at 359nm (I90C laser from Coherent). Blue emission was measured at 424nm (424/44 band pass filter) and red emission was above 675nm (675 AGLP long pass filter). All data was collected and analyzed with Summit™ Data Acquisition and Analysis Software (DakoCytomation).

Methylcellulose Stem Cell/Progenitor Cell Culture $2x10^4$ Side population cells were re-suspended in 2.5mL of Methocult media GF3434 (Stem Cell Technologies) using a 5mL syringe and a 12 gauge needle (Hierlihy et al., 2002). Cells were then plated on 2cm plastic petri dishes and incubated in humidity chambers at 37°C and

5% CO2 for 14days. At 14days post plating, colonies were counted using a Zeiss inverted microscope.

Statistical Analysis Differences between Ad-CT-1 and Ad-CTRL injected samples were evaluated for statistical significance using one tailed, unpaired Student's *t* test. Differences were considered statistically significant at a p value less than 0.05.

Results

CT-1 represses myogenic differentiation

Major sites of CT-1 expression during embryonic development are heart and skeletal muscle (Sheng et al., 1996). While CT-1's role in the cardiovascular system is being defined (Sheng et al., 1997), its role in skeletal muscle is not characterized. To begin to elucidate CT-1 function in skeletal muscle, we initially treated C2C12 cells chronically with CT-1 (10ng/ml, 0.5nM) and assessed muscle differentiation by the formation of multi-nucleated myotubes and accumulation of a skeletal muscle differentiation marker protein, MyHC. Solvent treated C2C12 cells began to exhibit multinucleated myotubes after 48hrs in DM. Thereafter, the control, solvent treated C2C12 cells developed MyHC-positive myotubes with large numbers of nuclei at later time points (figure 1A). In contrast, C2C12 cells in the CT-1 containing DM failed to form multinucleated myotubes at 48hrs. At later time points, some

myogenesis occurred although the number and calibre of MyHC-positive myotubes were greatly reduced in the presence of CT-1 compared to the corresponding controls (figure 1A). In addition, the MCK promoter activity was strongly inhibited by CT-1 as indicated by the transfection of a MCK promoter-reporter gene fused to enhanced signal Green Fluorescent Protein (EGFP) (pMCK-EGFP) (figure 1B). We also observed that CT-1 did not affect the cellular proliferation rate of differentiating myoblasts in DM assessed by BrdU incorporation rate (figure 1C&D). Therefore, these data document that CT-1 represses the skeletal muscle differentiation programme without affecting proliferation rate.

CT-1 represses the expression of pro-differentiation transcriptional regulators (MyoG and MEF2A/D)

To generate multi-nucleated myotubes from mono-nucleated myoblasts, the MRFs and MEF2s play an essential synergistic role at various stages of the differentiation programme (Kaushal et al., 1994). Therefore, we postulated that CT-1 might interfere with muscle differentiation through the MRFs and/or MEF2. First, in order to establish that the repression of myogenesis by CT-1 was observed in this analysis the levels of MyHC, a structural marker of muscle differentiation were assessed. As we expected that MyHC accumulated in the solvent treated C2C12 cells at late time points. Conversely, this accumulation of MyHC was largely attenuated in C2C12 cells treated with CT-1 (figure 2). Having determined that myogenesis was repressed by CT-1 at the molecular level, we next assessed the levels of various muscle transcription factors. Under these conditions, the expression of MyoG, a key MRF required for differentiation (Myer et al., 1997) was repressed by CT-1 compared to

the solvent treated cells, in which it was strongly induced (figure 2). In addition, MEF2A and MEF2D were also lower in the cells treated with CT-1 (figure 2). These data indicate that CT-1 inhibits myogenic differentiation by interfering with the up-regulation of MyoG and MEF2 factors. Interestingly, MyoD and Myf5 protein levels were relatively not affected by CT-1 suggesting that the lesion in the hierarchical differentiation programme lies between the MRFs required for lineage commitment (MyoD and Myf5) and the pro-differentiation transcriptional regulators (MyoG and MEF2A and MEF2D).

Transcriptional induction of the myoG promoter by MyoD is repressed by CT-1 signaling

Since MyoD, along with Myf5, play an early 'commitment' role in the myogenic cascade and also play an important role in the induction of the *myoG* gene (de la Serna et al., 2005), we hypothesised that CT-1 might interfere with the *trans*-activation properties of MyoD and therefore its ability to activate *myoG* transcription. To begin to address this hypothesis, we initially measured *myoG* promoter activity using reporter gene assays. In the absence of CT-1, the *myoG* promoter was activated in differentiating C2C12 cells in DM (figure 3). In the presence of CT-1, the activation of the *myoG* promoter was markedly inhibited in a dose dependent manner (figure 3). These data indicate that reduced MyoG levels observed with CT-1 (figure 2) result from a loss of transcriptional induction of the *myoG* locus.

Trans-activation properties of the MRFs are repressed by CT-1

Based on our observation that *myoG* gene transcription was attenuated by CT-1, we next focused on whether MyoD *trans*-activation properties might be altered by CT-1 since MyoD expression levels remained unaffected with CT-1 treatment (figure 2). The *trans*-activation capacity of MyoD has already been documented to be a heavily regulated aspect of its function, both positively and negatively, by a variety of mechanisms (Kim et al., 2008; Polesskaya et al., 2001; Puri et al., 2001; Reynaud et al., 2000). Bioinformatic analysis of MyoD interacting proteins revealed that MEK1 (Perry et al., 2001) and STAT3 (Kataoka et al., 2003) also share the property that they are known to be activated by phosphorylation in the presence of IL-6 family cytokines in different cell types. Therefore, we first confirmed that expression of CT-1 and its signal transduction receptors, Gp130 and LIFRβ in C2C12. Endogenous CT-1 and their receptor expression were confirmed by semi-quantitative RT-PCR analysis in the presence or absence of recombinant exogenous CT-1 in the media. We detected their transcripts in C2C12 cells, and their expression levels were not affected in the presence of CT-1 (figure 4A). We then surveyed these signaling molecules first by determining the phosphorylation levels of MEK1 and STAT3 in C2C12 cells acutely (figure 4B) or chronically (figure 4C) treated with CT-1 by Western blotting analysis. Indeed, levels of phosphorylated MEK-1 and STAT3 proteins in C2C12 cells were elevated in the presence of CT-1 compared to those in solvent control cells (figure 4B and C). A previous study indicated that MyoD's transcriptional activation properties can be inhibited by a direct interaction with MEK1 (Perry et al., 2001). Therefore, we sought to test this interaction by co-immuno-precipitation analysis.

These experiments revealed that exogenous expression of an activated form of MEK1 (Act-MEK: MEK1 R4F) and MyoD resulted in co-purification of the two molecules in the same complex, suggesting the possibility that this interaction can occur (figure 4D). In addition we observed that the typical activation of the *myoG* promoter (figure 4E) and MCK promoter (supplemental figure 1) by exogenously expressed MRFs was repressed by CT- 1 signaling (recombinant CT-1 or Act-MEK1) (figure 4E). These results further support the idea that MyoD *trans*-activation properties are repressed by CT-1 and that MEK activation is a key component of that repression.

CT-1 inhibits the transcriptional properties of the MRFs through activation of MEK signaling

To directly test the idea that CT-1 activation of MEK is responsible for MyoD *trans*-repression, we utilized MEK specific inhibitors, PD98056 and U0126. First, we reasoned that if MEK activation is absolutely required for CT-1 repression of myogenesis, then we should abrogate CT-1 effects on myogenesis by repression of MEK. In the absence of CT-1 (solvent), C2C12 cells formed multinucleated myotubes, and they accumulated MyHC proteins (brown colour) after 2days in DM (figure 5A). These morphological changes were not observed in the presence of CT-1. However, addition of MEK inhibitors neutralized the inhibitory effect of CT-1 on both myotube formation and MyHC accumulation in a dose dependent manner (3μM vs. 10μM) assessed by immunochemistry (figure 5A). Western blotting analysis of MyHC levels further confirmed the above observations (figure 5B). In agreement with this, a more detailed Western blotting analysis showed that MyoG protein levels were lower in the presence of CT-1, and this inhibitory effect was reversed by MEK

inhibition (PD98059), which prevented CT-1 mediated induction of phosphorylation of ERK (a MEK activity indicator). It was noted that as previously reported in different systems (Chen and Sytkowski, 2004; Yip-Schneider et al., 2009), the MEK inhibitor caused hyper-phosphorylation of MEK. However, in the presence of PD98059 upregulation of phospho-ERK by CT-1 was clearly inhibited (figure 5C). Therefore, this MEK inhibitor prevents CT-1 mediated activation of MEK. We also noticed that the MEK inhibitor reversed these CT-1 effects without affecting the phosphorylation levels of STAT3 (figure 5C, see below). Furthermore, luciferase reporter gene assays also showed that *myoG* promoter activity driven by exogenously expressed MyoD was repressed by CT-1. Furthermore,
exogenous expression of an activated form of MEK1 or Raf (components of the MAPK signaling pathway), also repressed *myoG* activation and these effects were reversed in a dose dependent manner by MEK inhibition (figure 5D), and by expression of dominant negative form of MEK1 or Raf1 (figure 5E). Therefore, these data indicate that MEK inhibition 'rescues' muscle differentiation from the inhibitory effect of CT-1, both morphologically and biochemically; and repression of MyoD's *trans*-activation properties by CT-1 is also reversed by MEK inhibition. Taken together, CT-1 represses skeletal myogenic differentiation through interference of the transcriptional activity of MyoD by the activation of MEK signaling.

STAT3 activation by CT-1 is not sufficient for inhibition of myogenesis

We documented that STAT3 is highly phosphorylated at tyrosine 705 (Y705) and serine 727 (S727) in response to CT-1 treatment (figure 4A&B). The Y705 phosphorylation is required for STAT3 dimer formation, nuclear translocation, and

transcriptional regulatory activity of STAT3 (Bromberg et al., 1999; Wen et al., 1995; Yu et al., 1995). Since a previous study showed that activated STAT3 can inhibit the transcriptional properties of MyoD (Kataoka et al., 2003), we postulated that STAT3 might also be involved in the repression of MyoD by CT-1 signaling. Western blotting analysis showed that the MEK inhibitor inhibited phospho-ERK1/2 (an indicator of MEK activity) activation by CT-1. However, MEK inhibition had no apparent effect on the phosphorylation levels of STAT3 at Y705 or S727 by acute or chronic CT-1 treatment (figure 5C and 6A). Since MEK inhibition rescues myogenic repression but does not alter STAT3 phosphorylation by CT-1, this indicates that STAT3 activation is not sufficient to inhibit myogenesis. To further address this issue, we next used a pan-JAK kinase inhibitor, P6 (Pedranzini et al., 2006) since STAT3 is phosphorylated by the Gp130/LIFRβ associated JAK kinases. As previously observed, CT-1 inhibited myotube formation and MyHC accumulation in DM compared to controls (figure 6B). In assessing the dose dependency of the P6, we observed no effect on CT-1 mediated myogenic repression up to a concentration at 250nM. However, at 500nM, P6 clearly neutralised the inhibitory effect of CT-1. Since P6 inhibits tyrosine kinase activity of other kinases at high levels (Thompson et al., 2002), we assessed the inhibitory effect of P6 on the phosphorylation levels of, STAT3, MEK1/2, and ERK1/2 by Western blotting analysis. As is claimed for this inhibitor, increased phosphorylation of STAT3 (Y705 and S727) by CT-1 was inhibited by P6 in a dose dependent manner (figure 6C). However, at a high concentration (500nM), P6 also repressed phosphorylation of ERK1/2 in the presence of CT-1. Since significant repression of phosphorylation of STAT3 was seen with the

P6 inhibitor at low concentrations (up to 250nM), but such concentrations had no effect in reversing CT-1 effects on myogenesis, we conclude that STAT3 activation by CT-1 is not sufficient to inhibit myogenesis. In agreement with the above results, MyoD driven *myoG* promoter activity was clearly inhibited in the presence of CT-1 (figure 6D). However, at any concentration tested, P6 had little effect on CT-1's inhibitory effect. In addition, exogenous expression of constitutively active (A662C and N664C) (Bromberg et al., 1999), phospho-mimetic mutant (Y705D and S727D) or dominant negative forms of STAT3 (S705F and S727A) (Kaptein et al., 1996) had no apparent effect on myogenesis phenotypically and biochemically in the presence of CT-1 (data not shown). Therefore, these results indicate that inhibition of MEK1/2 activity but not JAK activity is required for reversing the inhibitory effect of CT-1 on myogenesis. Taken together, we conclude that CT-1 inhibits skeletal muscle differentiation primarily through activation of MEK and, surprisingly, does not require STAT3 activation.

CT-1 increases the number of muscle precursor cells and delays regeneration of damaged muscle in vivo

To test the effect of CT-1 on *in vivo* skeletal muscle function, we utilized systemic delivery of a CT-1 expressing adenovirus, AdCT-1 (Bordet et al., 1999). AdCT-1 infection causes accumulation of CT-1 protein in cell-culture medium (figure 7A), and AdCT-1 injection leads to accumulation of CT-1 in liver and skeletal muscle (figure 7B). Although it did not lead to gross morphologic alterations in skeletal muscle (figure 7C), we noted a significant increase in the number of DAPI positive nuclei per myofiber following exposure to AdCT-1 compared to control injected

animals (Figure 7C&D; $P<0.05$). This observation suggested that CT-1 exposure represses differentiation leading to an increase in the number of undifferentiated myogenic precursors *in vivo*, similar to the effect elicited in C2C12 myoblast cell cultures. To test the possibility that CT-1 elicited an expansion of the myoblast/muscle precursor cell population, we also investigated the impact of CT-1 administration on the endogenous skeletal muscle progenitor pool. Skeletal muscle contains a population of cells that retain stem cell/progenitor like characteristics and these cells can be isolated based on Hoechst dye exclusion, referred to as side population (SP) cells (Asakura et al., 2002; Jackson et al., 2002; Muskiewicz et al., 2005). Skeletal muscle derived muscle progenitor cells from CT-1 injected animals were substantially increased compared to the number of progenitor cell colonies derived from control injected animals (10.6 vs 1.0; $P<0.05$; $n=7$). Based on our *in vitro* observations, we postulated that CT-1 exposure might also limit the differentiation of myoblasts *in vivo*. To test this supposition we induced muscle regeneration via cardiotoxin (CTX) injection in animals that received either AdCT-1 or the control adenovirus. CTX injury elicits a well defined response in which the myofibers are damaged, followed by expansion and differentiation of myogenic precursors to renew or replace the lost myofibers. Interestingly, CT-1 injected animals displayed a limited regeneration, exemplified by a marked reduction in the number of myofibers with centrally located nuclei and an expansion of mononucleated cells associated with regenerating myofibers compared to controls (Figure 7E). These results suggest that CT-1 targets myoblasts/muscle progenitor cells *in vivo* and actively represses the differentiation program. Taken together, our

results implicate a role for CT-1 in the maintenance of the undifferentiated state in muscle progenitor cells.

Discussion

In this study, we have characterised CT-1 as a potent inhibitory cytokine for the skeletal muscle differentiation program. We document that CT-1 activates MEK, which functionally abrogates the transcriptional activation properties of MyoD, a master regulator of myogenesis. Repression of this core muscle transcriptional network extinguishes induction of the *myoG* gene, an essential downstream regulator of the muscle differentiation program. Inhibition of muscle differentiation by CT-1 is MEK dependent since well-established MEK specific inhibitors, PD98059 and U0126 reverse CT-1's inhibitory effects on myogenesis both biochemically and phenotypically. Conversely, even though STAT3 is highly phosphorylated in the presence of CT-1, our experiments indicate that the phosphorylated STAT3 at Y705 and S727 is not sufficient to inhibit myogenesis. Thus, we conclude that CT-1 mediated inhibition of myogenesis requires MEK activation which subsequently interferes with the *trans*-activation properties of MyoD. This repression is independent of JAK-STAT signaling since pharmacological blockade of this pathway has no effect on the repression of myogenesis by CT-1.

Is MEK-ERK signaling a convergent regulatory nexus for cytokine mediated myogenic repression?

Several cytokines and growth factors such as FGF and EGF inhibit myogenesis through activation of MEK-ERK signaling. There are however some exceptions, such as IGF and Insulin, which activate MEK-ERK but paradoxically enhance muscle differentiation under some conditions. Since IGFs and Insulin also activate the Phosphatidy Inositol 3-Kinase (PI3K)-Akt pathway, and inhibition of PI3K or Akt neutralises their effect on myogenesis (Xu and Wu, 2000), it is likely that inhibition of differentiation is a "ground state" which can be overcome by pro-myogenic signals such as those mediated by Akt (Serra et al., 2007). This is essentially the sequence of events during ontogeny in which the muscle progenitor cells are held in an undifferentiated state until appropriate cues and conditions for differentiation are established. Thus, the dominance of pro-myogenic over inhibitory signals is a prerequisite for differentiation to occur. There is now substantial evidence suggesting that MEK activation is a point of convergence for several growth factors in repressing myogenesis (Page et al., 2004; Ramocki et al., 1997; Rommel et al., 1999). Evidence to date indicates that an activated nuclear MEK interacts with the MRFs and inhibits their transcriptional activation properties (Perry et al., 2001). The MRFs have consensus MAPK phosphorylation sites. However, MEK is capable of inhibiting the activity of a mutated form of Myf5, which does not have intact ERK phospho-acceptor sites. Therefore, the phosphorylation of the MRFs by MEK is not necessarily required for the repression (Winter and Arnold, 2000). Recently, transcriptional regulators have been found to recruit kinases in a stable manner to target promoters to

phosphorylate other components at the transcriptional machinery (Puri et al., 2001). Therefore, it is possible that the recruitment of kinases to muscle promoters is required for the inhibitory effects on differentiation. This is consistent with our data which indicates that the physical association of MyoD with MEK is crucial for the anti-myogenic activity of CT-1.

Interestingly, another member of the IL-6 cytokine family, LIF, was shown to inhibit skeletal myogenesis *in vitro* (Jo et al., 2005). In agreement with our observations, LIF mediated repression was also correlated with MEK-ERK pathway activation (Jo et al., 2005). LIF and CT-1 transduce signals in a similar manner through β-receptors such as Gp130 and LIFRβ. Prior to binding to the β-receptors, at least some of the IL-6 family cytokines bind to ligand specific α-receptors, and expression levels of the α-receptor in some cell types is known to regulate the sensitivity of the responsiveness to the specific ligand. Although LIF appears to bind β-receptors directly, CT-1 forms a complex with an α-receptor (Heinrich et al., 2003). However, this receptor has so far not been fully characterized, so a tissue specific role of this receptor has yet to be determined. In C2C12 skeletal muscle cells, we have confirmed that Gp130 and LIFRβ are expressed, and further characterization of the CT-1 α-receptor will delineate the precise receptor system. The convergence of LIF and CT-1 on MEK-ERK signaling suggests that this is a common nodal point for Gp130 linked cytokines.

CT-1 was originally isolated as a hypertrophic factor for cardiomyocytes *in vitro* (Pennica et al., 1995). Chronic administration of CT-1 into the mouse, indeed, causes hypertrophic hearts and also increases the size of liver, kidney, and spleen. This is, at least partially, the result of induction of the Vascular Endothelial Growth Factor

(VEGF) gene in cardiac myocytes through activation of the Gp130-JAK-STAT3 pathway (Jin et al., 1996). In addition, CT-1 activates MAPK pathways and the Akt-PI3 kinase pathway and protects cardiomyocytes from apoptosis (Brar et al., 2001; Liao et al., 2002; Sheng et al., 1997). One of the target genes of CT-1 in this cardio-protective role is the Small Proline-Rich Repeat protein-1A (SPRR1A) gene. CT-1 induces SPRR1A expression transcriptionally through activation of MEK-AP-1 and CCAAT/Enhancer-Binding Protein-β (C/EBPβ) pathways. This SPRR1A gene induction by CT-1 is independent of STAT3 activity but blunted by small chemical inhibitors of MEK activity, PD98059 and U0126 (Pradervand et al., 2004). Therefore, in other systems, CT-1 activates MEK kinases and regulates their down stream transcription factors. In C2C12 cells, we observed that the SPRR1A promoter was also up-regulated by CT-1 or an activated form of MEK, and this induction was dependent on MEK activation. However, SPRR1A over-expression does not inhibit myogenesis suggesting that this CT-1 target gene is not responsible for myogenic repression (data not shown). Our observations are discordant with a previous study, in which it was shown that activated STAT3 and MyoD physically interact and functionally antagonise each other by competing for limited amounts of co-activators, such as P300 and PCAF (Kataoka et al., 2003). We document that the pan-JAK inhibitor, P6, reduced the phosphorylation levels of STAT3 (Y705 and S727) by CT-1 but had little effect on myotube formation or MyoD's transcriptional activity at the concentration at which P6 inhibits phosphorylation of STAT3. Therefore, while we do not completely rule out the possibility that the inhibition of MyoD activity may be partly mediated by STAT3, we conclude that activation of MEK but not STAT3 is the

primary molecular event responsible for CT-1's inhibitory effect on myogenesis. Further support for this idea was recently provided by the observation that STAT3 and JAK2 were shown to be required for muscle differentiation C2C12 (Wang et al., 2008). Thus, the notion that STAT3 also functions in an inhibitory manner is unlikely. In addition, we observed that a well established JAK2 inhibitor, AG490, inhibited muscle differentiation in a dose dependent manner as previously reported (supplemental figure 2A) (Wang et al., 2008). However, this JAK2 inhibitor surprisingly had no effect on phosphorylation of STAT3 by CT-1 (supplemental figure 2B). Therefore, the JAK/STAT pathway does not appear to play a repressive role during myogenesis.

Given the temporal and spatial patterns of CT-1 expression during myogenesis, a pervasive consideration is whether CT-1 plays a role in the maintenance of the undifferentiated state or even pluripotency of progenitor cells in an autocrine or paracrine manner. Since CT-1 is expressed in skeletal muscle at key times during embryogenesis, and, as we observed, has a potent role in which it can reversibly repress myogenesis *in vitro* and delay regeneration *in vivo*. The observed *in vitro* and *in vivo* role of CT-1 in skeletal muscle cells defines it as a potential target of therapeutic interventions in which small molecule cell permeable inhibitors can be used to manipulate pro- and anti-differentiation pathways. Moreover, knowledge of these pathways could be instrumental in *ex-vivo* programming of progenitor cells which may have critical implications for a variety of cellular based muscle therapies.

In summary, we have documented that the CT-1 cytokine has a potent repressive effect on skeletal myogenesis *in vivo* and *in vitro*. This effect, which is reversible,

requires MEK-ERK signaling and, surprisingly, does not require STAT3 activation. The expression patterns of CT-1 and its *in vivo* and *in vitro* properties described here make it a viable candidate to play a role in the maintenance of the undifferentiated muscle progenitor cell state in embryonic and post-natal skeletal muscle.

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FIGURE LEGENDS

Figure 1. CT-1 represses myogenic differentiation. A) C2C12 cells were seeded onto cell culture plates at equal density and maintained in CT-1 (10ng/ml) or solvent containing growth medium (GM) or differentiation medium (DM) for the indicated time period. The cells were fixed and stained for muscle myosin heavy chain (MyHC) detection by immunochemistry. The photomicrographs are representative fields in each condition. B) C2C12 cells were plated at equal density and transfected with pCMV-deRed2 and pMCK-eGFP constructs. The transfected cells were maintained in CT-1 (10ng/ml) or solvent containing DM for 72 hrs to induce myotube formation. The cell morphology was recorded by phase-contrast microscopy and transfected cells were monitored by the red fluorescence signal. MCK promoter activity was assessed by the green fluorescence signal. C) C2C12 cells were maintained in DM for 72hrs and CT-1 (10ng/ml) or solvent was added every 24hrs. After 72hrs in low serum conditions cells were incubated with 100μM of BrdU for 1hr. Cells were then fixed with 70% ethanol and then incubated with 2N HCl to denature the DNA. The cells were then blocked with 10% goat serum prior to incubation with BrdU primary antibody. The cells were then washed with 1x PBS-T and incubated with secondary antibody conjugated to FITC. The cells were washed with 1x PBS-T and mounted using fluorescence mounting media and viewed under a fluorescence microscope. D) The average of percentage of BrdU positive nuclei over total nuclei in 12 individual fields per condition was calculated and graphed. (error = standard variation)

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Figure 2. CT-1 represses the expression of pro-differentiation transcriptional regulators (MyoG and MEF2A/D). C2C12 cells were induced to differentiate in DM with CT-1 (10ng/ml) or solvent. The cells were maintained in the indicated conditions for specific time periods. Total protein samples were extracted from the cells and equal amounts of total protein (20μg) were subjected to Western blotting analysis. The levels of indicated proteins were assessed by a standard immuno-blotting technique with a specific primary antibody. Actin indicates equal amounts of protein loading into each lane.

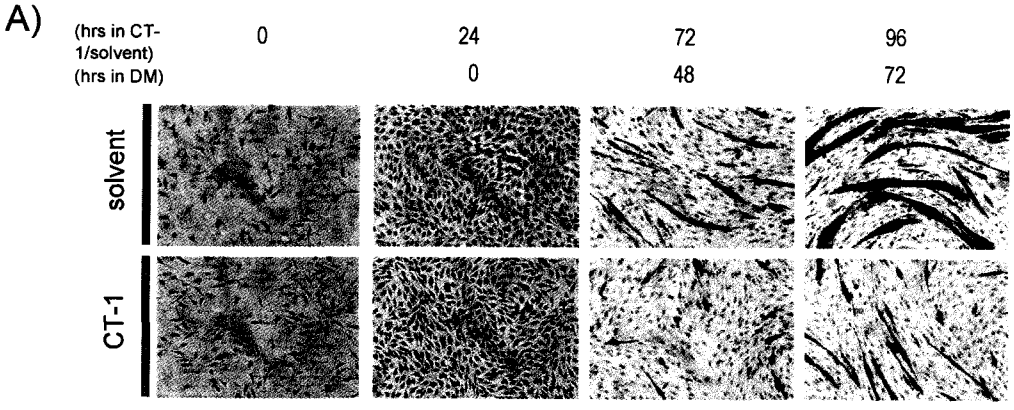
Figure 3. Transcriptional induction of the *myoG* promoter by MyoD is repressed by CT-1. C2C12 cells were transfected with either pGL3 (empty control) or a Myogenin promoter-luciferase reporter gene construct (pMyoG-Luc), and to monitor transfection efficiency, pCMV-β-gal construct was included in each condition. The transfected cells were maintained for 16 hrs in the indicated concentration of CT-1 or its solvent in DM. Total protein samples were harvested with a luciferase lysis buffer. Luciferase activity in each condition was measured independently and normalized according to β-Galactosidase activity.

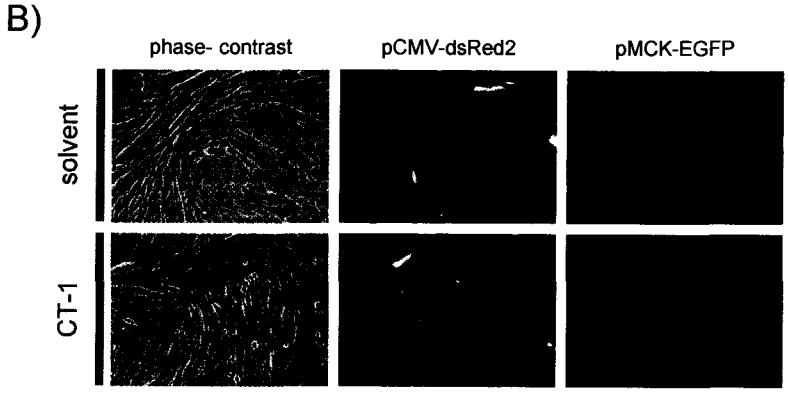
Figure 4. *Trans*-activation properties of the MRFs are repressed by CT-1. A) Total RNA was isolated from C2C12 cells in GM (lane 1), DM with solvent (48hrs) (lane 2), and DM with CT-1 (10ng/ml) (48hrs) (lane 3) and subjected to semi-quantitative RT-RCP analysis with indicated gene specific primer pairs. RT-PCR amplified DNA was separated in a TAE/agarose-gel, and EtBr stained DNA was visualised by UV irradiation. GAPDH serves as an internal loading control. B) C2C12 cells were plated at equal density and kept in DM for 16hrs. CT-1 (10ng/ml) or equal volume of the solvent was added to the media. The cells were harvested after 20min of CT-1/solvent addition. Total protein samples were subjected to Western blotting analysis to estimate the levels of indicated proteins. C) Western blotting analysis was performed as described above. However, the cells were maintained in DM with CT-1 or solvent for indicated time periods. D) C3H10T1/2 cells were transfected with combinations of the indicated constructs. Total protein samples were extracted from the cells maintained in DM. Exogenous-expression of MyoD and an activated form of MEK1 was confirmed by immuno-blotting (IB) (10μg loading) with the specific antibodies. An immuno-precipitation (IP) analysis was performed with the total protein extract (250μg) with MyoD antibody (mouse) and proteinG conjugated beads. Precipitated immuno-complex were eluted off the proteinG beads and subjected for an immunoblotting with MEK antibody (Rabbit). Equal amount of IgG loading was monitored with MyoD immuno-blotting with MyoD specific antibody (Rabbit). E) C2C12 cells were transfected with the indicated expression constructs or its empty vector (1μg) and *myoG* promoter-(pMyoG-Luc) promoter-Luciferase reporter construct (0.5μg). In addition, an activated form of MEK1 expression vector (1μg) or its empty vector (for CT-1 and solvent) was included. To monitor transfection efficiency, a pCMV-β-Gal construct was also included (0.3μg). After transfection, the cells were maintained in DM containing CT-1 (10ng/ml) or its solvent for 16 hrs. The cells were harvested and subjected to luciferase assay and β-Galactosidase assay.

Figure 5. CT-1 inhibits the transcriptional activity of the MRFs through activation of MEK signaling. A) C2C12 cells were plated at equal density and induced differentiation transferred into DM upon about reaching confluence. The cells were maintained in indicated concentration of MEK inhibitor (PD98059, U0126, or DMSO; 3μM or 10μM) with without CT-1 (10ng/ml). After 2days in the indicated conditions, the cells were fixed and stained for MyHC detection by immunochemistry with MF-20 mouse monoclonal antibody. MyHC protein accumulation was indicated by brown color. The photomicrographs are representative fields. B&C) C2C12 cells were maintained in DM with CT-1 (10ng/ml) and or PD98059 (10μM), or their solvents for 2days (C) or 3days (B) to induce myotube formation. Total cellular proteins were extracted from the cells in each condition. The total protein lysate samples (20μg) were subjected to Western blotting analysis. Actin levels indicate loading of an equal amount of the total protein into each lane. D) C2C12 cells were transfected with a pMyoG-Luc (0.5μg), a MyoD expression vector (1μg), a pCMV-β-Gal (0.3μg), and also the indicated kinase expression vector (act.MEK1, act.Raf) or an empty vector (1μg). The transfected cells were maintained in DM containing CT-1 (10ng/ml) or solvent, and the indicated concentration of PD98059 MEK inhibitors for 16hrs. The cells were harvested and subjected to Luciferase assay and β-Gal assay. Luciferase activity was normalized according to the β-galactosidase activity from a co-transfected pCMV-β-Gal expression construct by calculating the Relative Luciferase Unit (RLU) for each individual condition, and the fold-activation was calculated with respect to the average RLU of the "empty vector + solvent" at the corresponding concentration of PD98059. E) C2C12 cells were transfected with a pMyoG-Luc (0.5μg), a pCMV-β-Gal (0.3μg), and also the indicated kinase expression vector (DN-MEK1, DN-Raf1) or an empty vector (1μg). The transfected cells were maintained in DM containing CT-1 (10ng/ml) or solvent for 16hrs. The cells were harvested and subjected to Luciferase assay and β-Gal assay.

Figure 6. STAT3 activation by CT-1 is not sufficient for inhibition of myogenesis. A) C2C12 cells were plated at equal density maintained in DM. A MEK inhibitor (PD98059 (10μM)) or DMSO was added 30min before the addition of CT-1 (10ng/ml) or its solvent. After 20 min of CT-1 or solvent treatment, the cells were harvested, and total protein samples were extracted for each condition. The protein samples (20μg) were subjected to Western blotting analysis. B) An equal number of C2C12 cells were plated and maintained in DM containing CT-1 (10ng/ml) or its solvent, in addition, the indicated concentration of pan-JAK kinase inhibitor, P6, was included in the DM. The cells were fixed after maintained in the DM for 3days, and accumulation of MyHC was visualized by immunochemistry. The brown color indicates MyHC accumulation in the cells. The photomicrographs are representative fields of each condition. C) C2C12 cells were plated at equal density and maintained in DM for 16 hrs. Thirty min before adding CT-1 (10ng/ml) or its solvent, the cells were treated with indicated concentration of P6 (pan-JAK kinase inhibitor). After 20 min of CT-1 or solvent addition to the media, the cells were harvested. Total protein samples were extracted from the cells in each condition, and equal amounts of the protein (20μg) was subjected for Western blotting analysis. D) C2C12 cells were transfected with an either pMyoG-Luc or pGL4-10 (0.5μg), and a MyoD expression vector (1μg), a pCMV-β-Gal (0.3μg). The transfected cells were maintained in DM containing CT-1 (10ng/ml) or solvent, and the indicated concentration of P6 pan-JAK inhibitor for 16hrs. The cells were harvested and subjected to Luciferase assay and β-Gal assay.

Figure 7. CT-1 delays regeneration of damaged skeletal muscle *in vivo* A) Immunoblotting was used to verify the efficacy of Adenovirus CT-1 production. Recombinant CT-1 protein (100ng) was used as a positive control, as well as media from CT-1 adenovirus infected myocytes. At 72hrs post-infection, the media from the treated and untreated cells was collected and subjected to Western blotting analysis with a CT-1 antibody. B) At 7 days post-injection, skeletal muscle (sk.muscle) and liver samples were excised from adenovirus injected mice. Frozen tissue was homogenized and a total of 300μg of protein was electrophoresed on a 15% SDS-PAGE. An equal protein loading was verified by Western blotting analysis using β-tubulin specific antibody C) At 7days post-injection, skeletal muscle was excised, fixed then embedded in paraffin, and sectioned at 10μm. These sections were counterstained with haematoxylin and eosin to visualize the nuclei and cytoplasm. For immuno-histological detection of β-actinin, the sections were incubated with β-actinin antibody (Abcam), then incubated in donkey-anti-goat CY3 antibody (Chemicon) and finally counterstained with DAPI (Sigma). The micrographs were representative fields D) 5 fields of view per section and 5 sections per TA muscle were analysed. Differences between Ad-CT-1 and Ad-CTRL injected samples were evaluated for statistical significance using one tailed, unpaired Student's *t* test. Differences were considered statistically significant at a p value less than 0.05. (n=3)
E) B6C3F1 mice were subject to systemic delivery of the CT-1 adenovirus. The injections were administered via intra-cardiac chamber delivery with 50μL of Ad-CT-1 at a concentration of 3.0 X 10^8 PFU/mL (n=3). A control group of B6C3F1 mice were injected with 50 μL Ad-CTRL at a similar concentration (n=3). In a separate group of animals, cardiotoxin was used to induce muscle injury immediately prior to AdCT-1 and Ad-CTRL injection (n=3 for each group). 25μl of 10μM cardiotoxin (Latoxan) was injected directly into the TA muscle. 5 fields of view per section and 5 sections per TA muscle were analysed. The micrographs were representative views. During post-recovery, mice were monitored closely for weight loss, dehydration and cardiac distress.


A)
(hrs in CT-1/solvent)
(hrs in DM)
0
24
0
72
48
96
72
solvent
CT-1


B)
phase- contrast
pCMV-dsRed2
pMCK-EGFP
solvent
CT-1

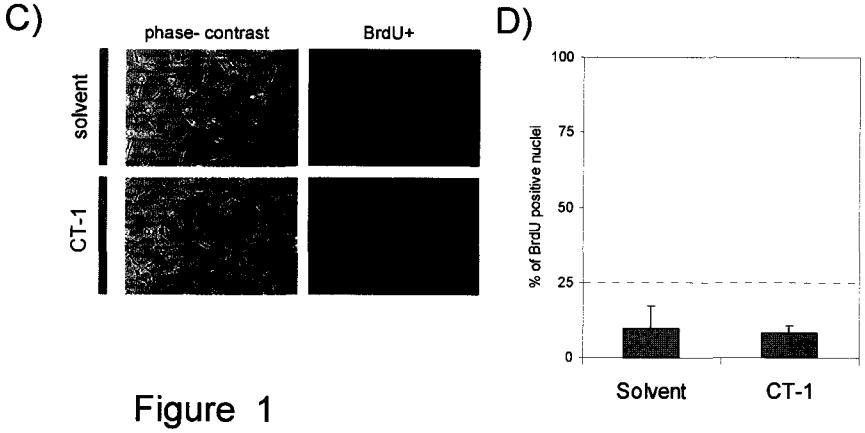

C)
phase- contrast
BrdU+
solvent
CT-1
D)
100
75
50
25
0
% of BrdU positive nuclei
Solvent
CT-1

Figure 1

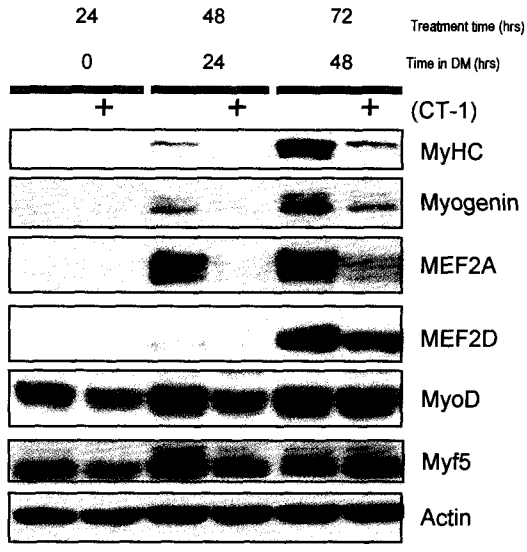


Figure 2

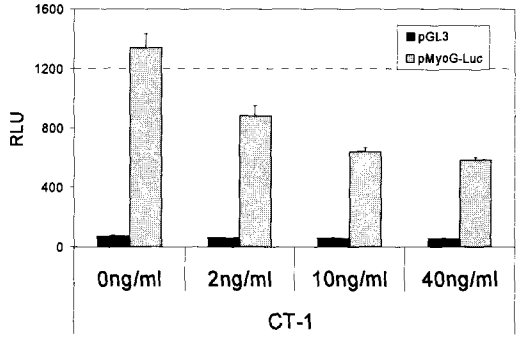


Figure 3

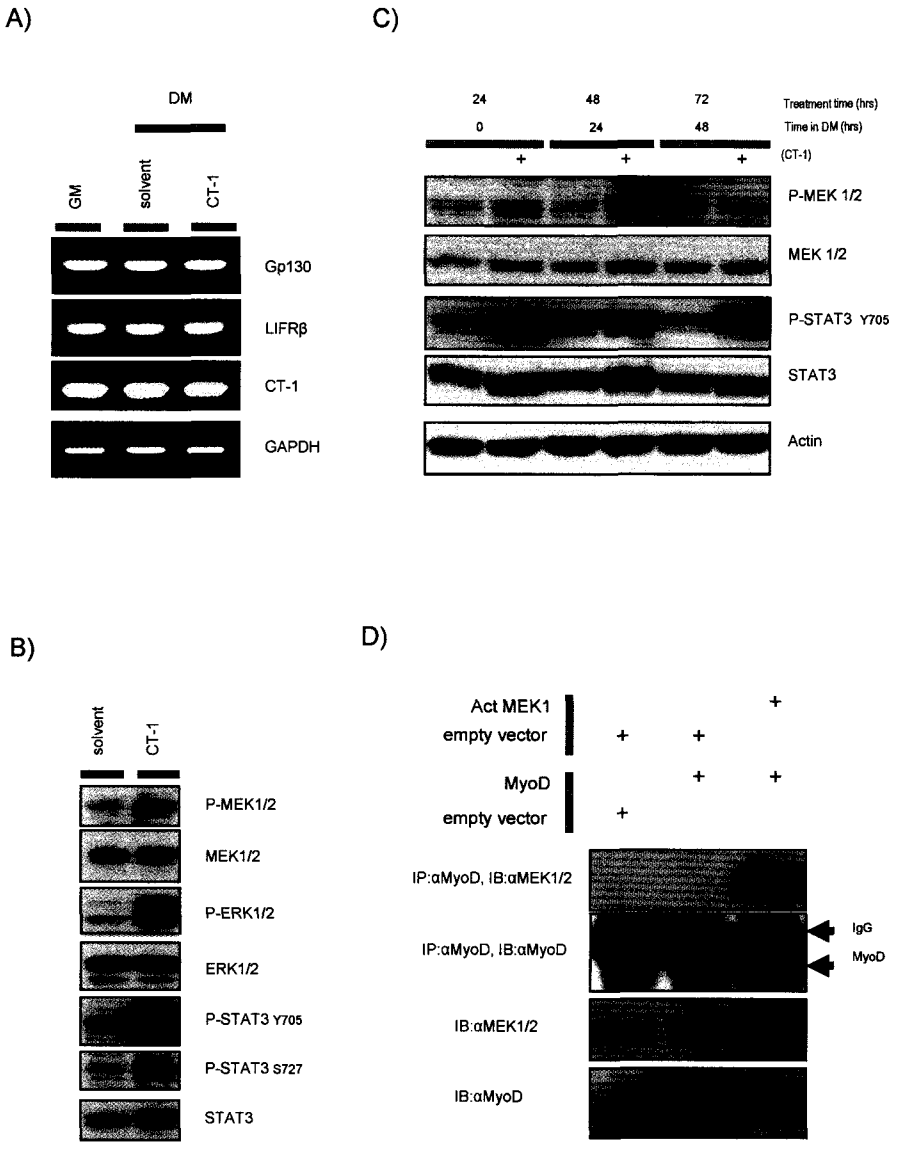

A)
DM
GM
solvent
CT-1
Gp130
LIFRβ
CT-1
GAPDH
C)
24
48
72
Treatment time (hrs)
0
24
48
Time in DM (hrs)
(CT-1)
+
+
+
P-MEK 1/2
MEK 1/2
P-STAT3 Y705
STAT3
Actin
B)
solvent
CT-1
P-MEK1/2
MEK1/2
P-ERK1/2
ERK1/2
P-STAT3 Y705
P-STAT3 S727
STAT3
D)
Act MEK1
empty vector
MyoD
empty vector
IP:αMyoD, IB:αMEK1/2
IP:αMyoD, IB:αMyoD
IgG
MyoD
IB:αMEK1/2
IB:αMyoD

Figure 4

E)

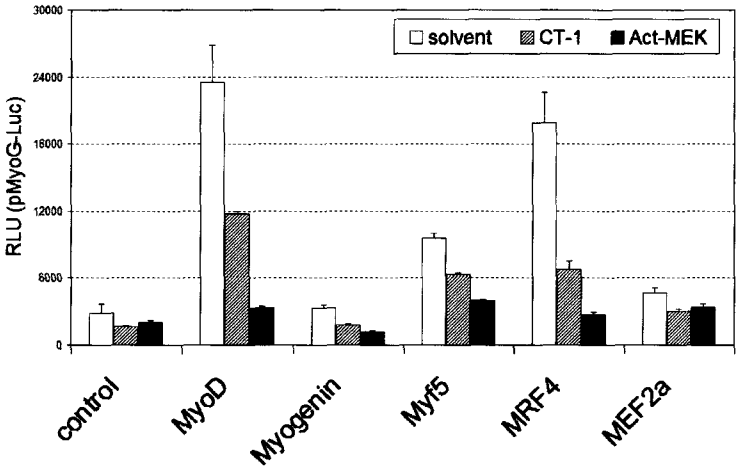
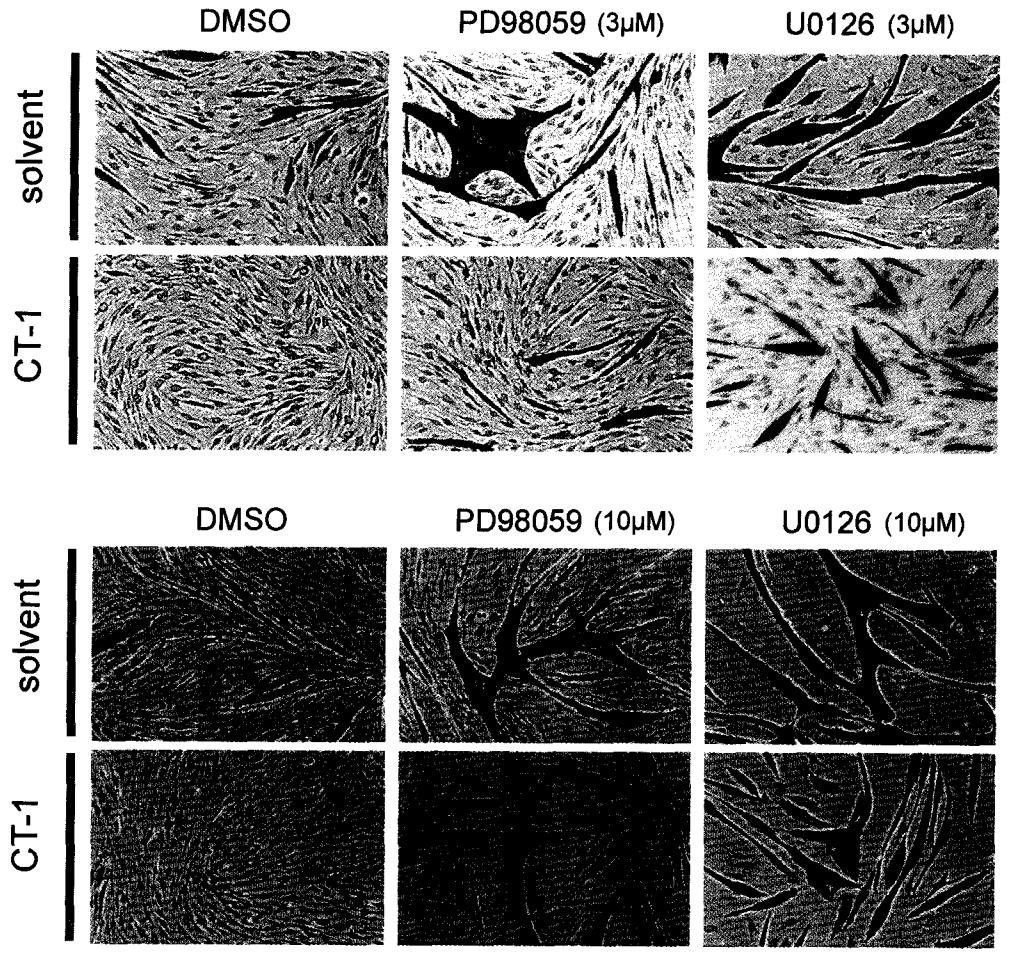

30000
24000
18000
12000
6000
0
RLU (pMyoG-Luc)
□ solvent
▨ CT-1
■ Act-MEK
control
MyoD
Myogenin
Myf5
MRF4
MEF2a

Figure 4

A)


DMSO
PD98059 (3μM)
U0126 (3μM)
solvent
CT-1
DMSO
PD98059 (10μM)
U0126 (10μM)
solvent
CT-1

B)

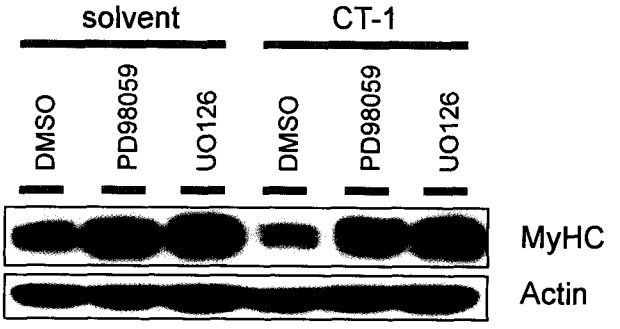
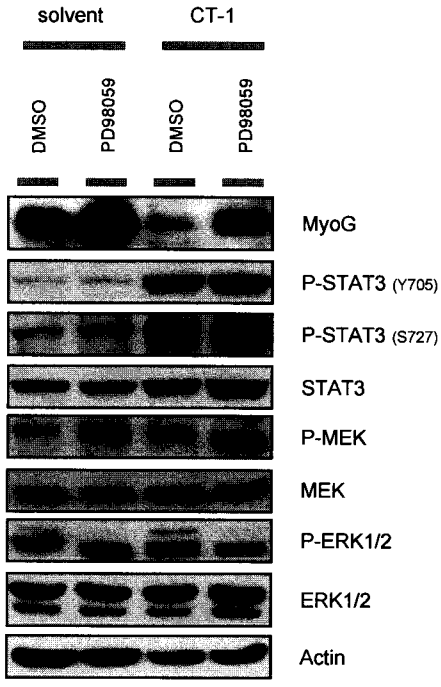
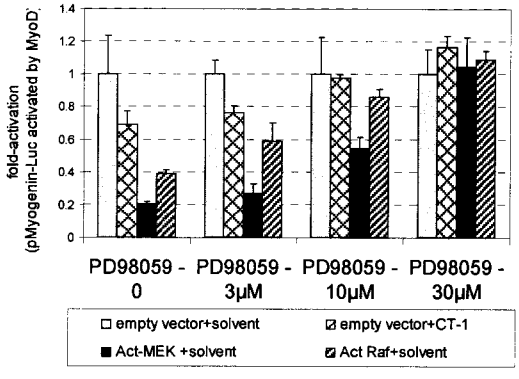

solvent
CT-1
DMSO
PD98059
UO126
DMSO
PD98059
UO126
MyHC
Actin

Figure 5

C)


solvent
CT-1
DMSO
PD98059
DMSO
PD98059
MyoG
P-STAT3 (Y705)
P-STAT3 (S727)
STAT3
P-MEK
MEK
P-ERK1/2
ERK1/2
Actin

D)


fold-activation
(pMyogenin-Luc activated by MyoD
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PD98059 - 0
PD98059 - 3μM
PD98059 - 10μM
PD98059 - 30μM
empty vector+solvent
empty vector+CT-1
Act-MEK +solvent
Act Raf+solvent

E)

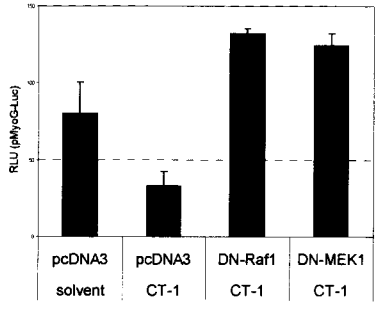

RLU (pMyoG-Luc)
pcDNA3
solvent
pcDNA3
CT-1
DN-Raf1
CT-1
DN-MEK1
CT-1

Figure 5

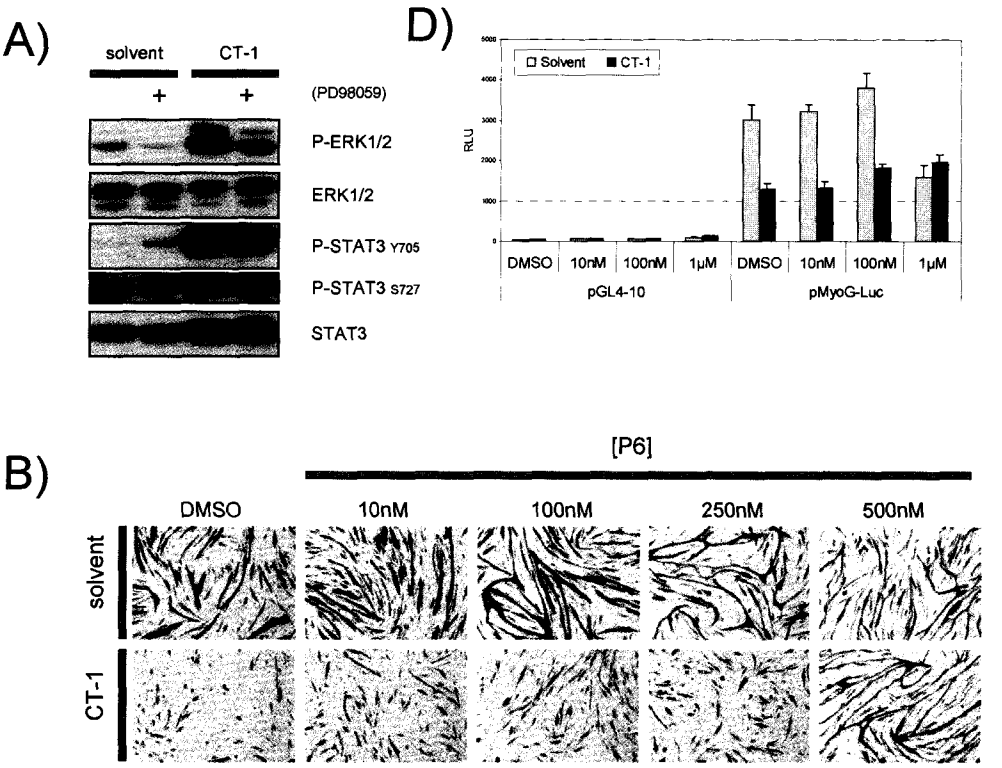

A)
solvent
CT-1
+
+
(PD98059)
P-ERK1/2
ERK1/2
P-STAT3 Y705
P-STAT3 S727
STAT3
D)
Solvent
CT-1
RLU
DMSO
10nM
100nM
1μM
DMSO
10nM
100nM
1μM
pGL4-10
pMyoG-Luc
B)
[P6]
DMSO
10nM
100nM
250nM
500nM
solvent
CT-1
C)
0
100
250
500
1000
([P6] (nM))
+
+
+
+
+
(CT-1)
P-STAT3 Y705
P-STAT3 S727
STAT3
P-MEK1/2
MEK1/2
P-ERK1/2
ERK1/2

Figure 6

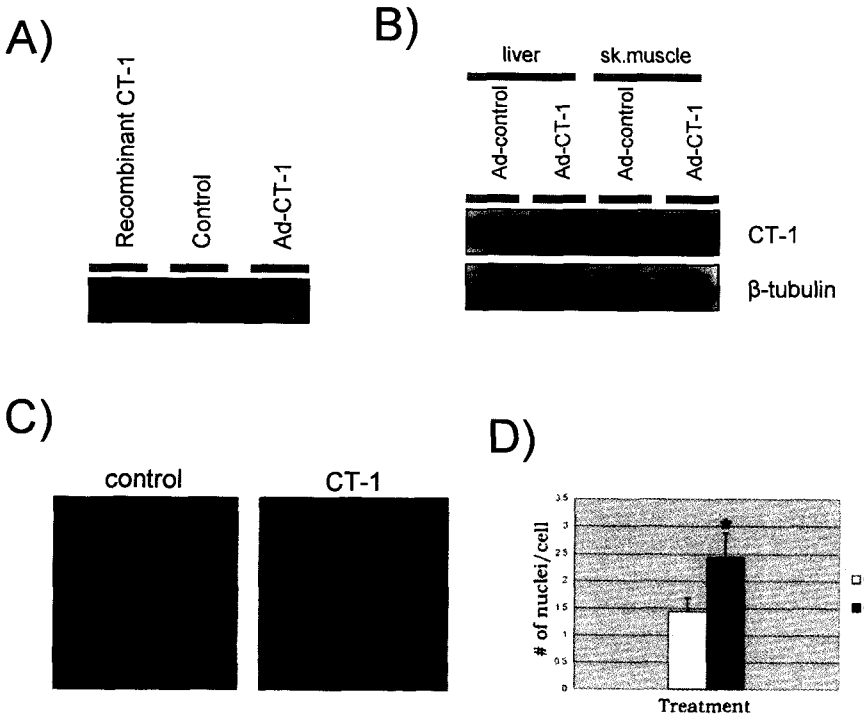
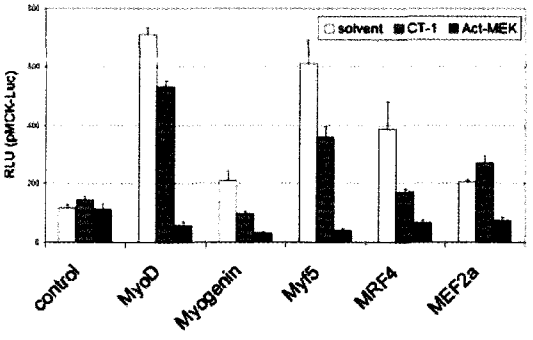
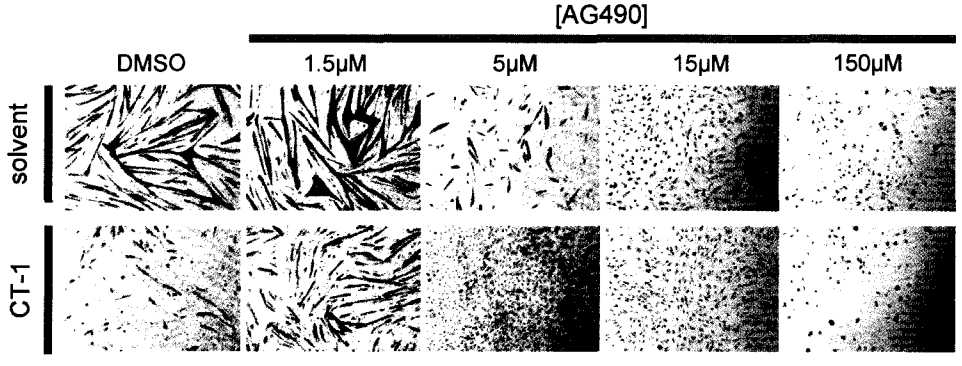

A)
Recombinant CT-1
Control
Ad-CT-1
B)
liver
sk.muscle
Ad-control
Ad-CT-1
Ad-control
Ad-CT-1
CT-1
β-tubulin
C)
control
CT-1
D)
# of nuclei/cell
Treatment
CTRL
CT-1
E)
Ad-control
Ad-CT-1

Figure 7

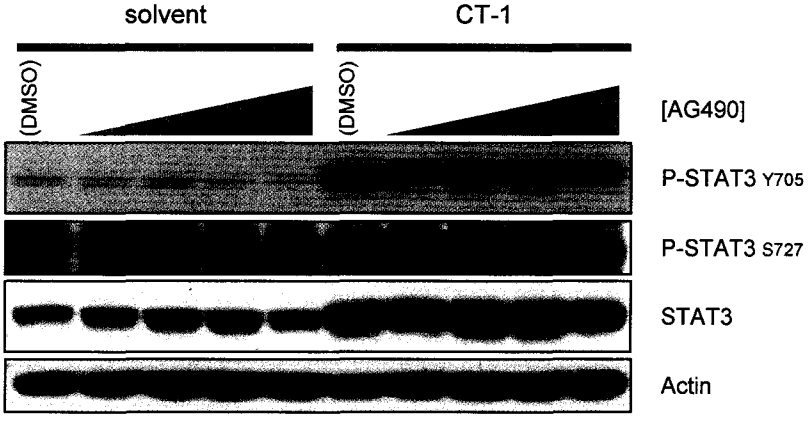


Supplemental figure 1

A)



B)



Supplemental figure 2

Chapter IV; Nuclear function of Smad7 promotes myogenesis

In Press (accepted) "Molecular and Cellular Biology" published ahead of print on 7 December 2009, doi:10.1128/MCB.01005-09

Experimental design and drafting manuscript
Tetsuaki Miyake and Dr. John C McDermott

Conducting experiments
Tetsuaki Miyake; (all figures except figure 5G)
Nezeka S Alli (figure 5G)

Nuclear function of Smad7 promotes myogenesis

Running title: Nuclear Smad7 promotes myogenesis

Tetsuaki Miyake, Nezeka S. Alli, and John C. McDermott*

Department of Biology, York University, 4700 Keele street, Toronto, Ontario, M3J 1P3 Canada.

*Corresponding author: Mail address, Department of Biology, 327 Farquharson, LSB, York University, 4700 Keele St., Toronto, Ontario, Canada. M3J 1P3

Phone: (416)-736-2100 ext.30389, Fax: (416)-736-5698, E-mail: jmcderm@yorku.ca

Abstract (199 words, 1174 characters)

Introduction (593 words, 3446 characters)

Materials and Methods (978 words, 5895 characters)

Results (2418 words, 13929 characters)

Discussion (637 words, 3761 characters)

Figure Legends (1989 words, 11743 characters)

Total (6814 words, 39948 characters)

Abstract

In the 'canonical' view of transforming growth factor-β (TGFβ) signaling, Smad7 serves an inhibitory role. While Smad7 represses Smad3 activation by TGFβ, it does not reverse the inhibitory effect of TGFβ on myogenesis suggesting a different function in myogenic cells. We previously reported a pro-myogenic role of Smad7 mediated by an interaction with MyoD. Based on this association, we hypothesized a possible nuclear function of Smad7 independent of its role at the level of the receptor. We therefore engineered a chimera of Smad7 with a nuclear localization signal (NLS), which serves to prevent, and therefore bypass, binding to the TGFβ receptor while concomitantly constitutively localizing Smad7 to the nucleus. This Smad7-NLS did not repress Smad3 activation by TGFβ but did retain its ability to enhance myogenic gene activation and phenotypic myogenesis indicating that the nuclear, receptor independent, function of Smad7 is sufficient to promote myogenesis. Furthermore, Smad7 physically interacts with MyoD and antagonizes the repressive effects of active-MEK on MyoD. Reporter and myogenic conversion assays indicate a pivotal regulation of MyoD transcriptional properties by the balance between Smad7 and active-MEK. Thus, Smad7 has a nuclear co-activator function that is independent of TGFβ signaling and necessary to promote myogenic differentiation.

Introduction

Skeletal muscle differentiation results from a highly orchestrated program of gene expression. Extensive biochemical and genetic evidence has implicated a family of DNA binding transcriptional regulatory proteins encoded by the *myogenic regulatory factor (MRF)* genes, *myf5, myod, myogenin (myog),* and *mrf4*, in this process. In conjunction with the proteins encoded by the *myocyte enhancer factor two* (MEF2A-D) gene family, the MRF's activate an evolutionarily conserved program of gene expression, which leads to the generation of terminally differentiated multinucleated myotubes from mononucleated precursor cells (10, 16, 22, 24, 40, 41, 45, 51). The transcriptional activation properties of the MRF and MEF2 complexes are potently regulated by diverse protein-protein interactions (4, 6, 25, 26, 30, 31, 35, 37, 43, 47, 49, 61) and myriad post-translational modifications (7, 9, 13, 23, 46, 54, 59, 60). This integrated network of protein complexes specifies a unique code for the establishment of myogenic lineage commitment and differentiation. The dynamic nature of these transcriptional regulatory complexes is acquired by an exquisite responsiveness to the milieu of cytokines and growth factors that regulate the myogenic cascade (1, 14, 19, 21, 38, 44, 50, 52, 55). Amongst a plethora of secreted soluble growth factors affecting muscle differentiation, TGFβ and Myostatin have been implicated as potent repressors of the myogenic gene expression program.

The TGFβ superfamily of cytokines has been shown to function through a 'canonical' pathway in which the receptor regulated Smads (R-Smads) transduce signals to the nucleus to modulate gene expression in response to ligand-receptor interaction. An interesting feature of this signal transduction cascade is the existence of inhibitory Smads

(I-Smad; Smad6 and Smad7) which serve to repress receptor mediated signaling in an auto-regulatory feedback loop. Smad7 is primarily characterized as a negative regulator of the TGFβ-Smad2/3 pathway (17). The 'canonical' view is that Smad7 prevents Smad2/3 from being phosphorylated by the TGFβ type I receptor (ALK5) by physical interaction with the cytoplasmic tail of the receptor complex; as a result, Smad7 inhibits Smad2/3 and Smad4 complex formation and subsequent nuclear accumulation of this complex (42, 56). Both TGFβ and Myostatin repress myogenesis *in vitro* and *in vivo* respectively (2, 3, 5, 15, 36, 57). These pathways converge on Smad2/3 through formation of activated receptor complexes with type I (ALK5) and II TGFβ receptors for TGFβ (18, 33, 34, 53, 58), and type I (ALK7) and IIB activin receptors for Myostatin (27, 28). Despite the commonality of this effecter system, we previously found that exogenous Smad7, which functions as an inhibitory Smad, reverses the inhibitory effect of Myostatin but not TGFβ on muscle differentiation suggesting that some aspects of the downstream signaling are divergent (26). In these studies, it was documented that Smad7 fulfills an essential and enhancing role for muscle differentiation. Preliminary evidence suggested that Smad7 might co-operate with a nuclear transcription factor in order to enhance muscle differentiation through potentiation of the transcriptional properties of MyoD (26).

In this report, we systematically dissect the role of Smad7 in the nucleus in myogenic cells. Constitutive nuclear localization of Smad7 was engineered by fusing a nuclear localization signal (NLS) to Smad7 (Smad7-NLS). Smad7-NLS accumulates in the nucleus, bypassing its 'canonical' ability to inhibit Smad3 activation by TGFβ. The nuclear Smad7 retains its capacity to enhance MyoD's transcriptional properties and

myogenic differentiation independent of its ability to abrogate Smad3 activation at the level of the receptor. In addition, we found that Smad7 antagonizes the repressive effects of MAP kinase kinase (MEK) on MyoD function. Collectively, these observations support a pro-myogenic role of nuclear Smad7 independent of its role in antagonizing TGFβ signaling.

Materials and Methods

Plasmids Smad7 and Smad7T expression vectors were described previously (26). The ORF of EGFP without a stop codon was inserted at the *HindIII* site of pcDNA3-Smad7 or pcDNA3-Smad7T expression vectors for expression of the fusion peptide, and the EGFP ORF with a stop codon was inserted at the *HindIII* site of pcDNA3 empty vector (invitrogen). NLS of SV40 (5'tc gag ggt gga ggt cc acct aaa aag aag cgg aaa gtg ggt gga ggt t 3' and 5'ct aga acc tcc acc cac ttt ccg ctt ctt ttt agg tgg acc tcc acc c 3') was inserted at the *XhoI/XbaI* site in frame to generate an EGFP-Smad7-NLS construct. MRF expression plasmids were constructed in pEMSV as described elsewhere (8). MyoD deletion constructs were a kind gift from S.Tapscott (Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle). An activated (ΔN3 S218D/S222E) human MEK1 expression construct was a gift from A.Natalie (32). The reporter construct pMCK-EGFP was provided by A. Ferrer-Martinez (Universitat de Barcelona, Spain). 3TP-Lux, and expression vectors for DN-ActII, DN-TβIIR were from J.Wrana (University of Toronto; Program in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute, Mount Sinai Hospital). The expression vector for DN-TβIIR was from

J.Massague (Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York). Smad 7 promoter-luciferase-reporter constructs were provided by S.Dooley (Molecular Alcohol Research in Gastroenterology, II, Medical Clinic, Medical Faculty Mannheim, University of Heidelberg, Heidelberg, Germany). (CAGA)X10-Luc reporter construct was generated by insertion of 10X (CAGA) sequence from the *pai-I* promoter (11) followed by a *c-fos* minimal promoter in the pGL3-basic (Promega) luciferase reporter vector. Transcription reporter constructs, pMCK-luc (12), pCMV-β-galactosidase were described elsewhere (26). The myogenin promoter region was excised from pMyoG-luc by *SacI /Bgl II* digestion. The resultant 1152bp fragment was inserted at the SacI */Bgl II* sites of pGL4-10 vector (Promega). The dsRed2-N1 expression vector was purchased from Clontech Laboratories. All constructs used in this study were verified by DNA-sequencing (York University Molecular Core Facility).

Antibodies The primary antibodies used in this study were obtained from Santa Cruz Biotechnology; MyoD (sc-304), GFP (sc-5385), Actin (sc-1616), c-Jun (sc1694), Myf5 (sc-302) from Cell Signaling Technology; MEK1/2 (9122), Phospho-MEK1/2 (Ser217/221) (9121), from Developmental Studies Hybridoma Bank; Myogenin (F5D), Myc (9E10), from DakoCytomation; MyoD1 (clone:5.8A; M3512), from R&D system; Smad7 (MAB2029). Normal mouse IgG (sc-2025) was from Santa Cruz Biotechnology.

Cell Culture C2C12 myoblast and C3H10T1/2 were obtained from American Type Culture Collection and cultured in growth medium (GM) consisting of 10% Fetal bovine serum (FBS) (HyClone) in high-glucose Dulbecco's modified Eagle's medium (DMEM)

(Gibco) supplemented with 1% penicillin-streptomycin (Gibco) at 37°C and 5% CO_2. Myotube formation was induced by replacing GM with differentiation medium (DM) which consisted of 2% horse serum (Atlanta Biologicals) in DMEM supplemented with 1% penicillin-streptomycin. For TGFβ or CT-1 treatment, recombinant human TGFβ (R&D system; 240-B) or CT-1 (R&D system; 438-CT) was resuspended with solvent (4mM HCl, 0.1% bovine serum albumin (BSA)) and added into the media. For myotube formation assays, DM with TGFβ (1ng/ml) or CT-1 (10ng/ml) was replenished every 2 days.

Microscopy and Fluorescence Fluorescence and phase contrast photomicrographs were obtained using an epifluoresence microscope (Axiovert 35; Carl Zeiss MicroImaging), with appropriate phase and filter settings, and either 4X NA 0.10 or 10X NA 0.25 Achrostigmat objective lenses. Images were recorded with a digital camera (Canon, EOS D60).

Nuclear protein extraction Nuclear proteins were extracted from the cells by NE-PER® kit (Pierce) according to the manufacturer's protocol.

Western blotting analysis Total cellular protein extracts were prepared in NP-40 lysis buffer (0.1 % NP-40, 150 mM NaCl, 1mM EDTA, 50 mM Tris-HCl pH 8.0, 1mM sodium vanadate, 1mM PMSF, supplemented with a protease inhibitor cocktail (Sigma, P-8340)). Protein concentrations were determined by a standard Bradford assay (BioRad). Equivalent amounts of protein were resolved by SDS-PAGE gels, followed by

electrophoretic transfer to an Immobilon-P membrane (Millipore) as directed by the manufacturer (Millipore). Blots were incubated with the indicated primary antibody in 5% milk in PBS or Tris buffered saline (TBS)-T (10mM Tris-HCl pH8.0, 150mM NaCl, 0.1% Tween-20) or 5% Bovine serum albumin (BSA) in TBS-T according to the manufacturer's protocol at 4°C overnight with gentle agitation. After washing briefly, the blots were incubated with the appropriate HRP-conjugated secondary antibodies in 5% milk in PBS or TBS-T at room temperature according to the manufacturer's protocols (Santa Cruz Biotechnology, Cell Signaling Technology). After being washed three times with 1XPBS or 1XTBS (depending on the primary antibody) at room temperature, the blots were treated with the Enhanced chemiluminescence reagent (Amersham) to detect immuno-reactive proteins. The blots were exposed to Biomax film (Kodak) for visual representation.

Transcription reporter gene assays C2C12 myoblasts were transfected by a standard calcium phosphate-DNA precipitation method with the indicated reporter gene and expression constructs and pCMV-β-galactosidase to monitor transfection efficiency. After transfection, the cells were washed with PBS and maintained in GM and then treated as indicated. Total cellular protein was extracted with luciferase lysis buffer (20mM Tris-HCl pH7.4, 0.1% Triton X-100). Luciferase and β-galactosidase enzyme assays were performed according to the manufacturer's protocol (Promega). Luciferase activity was quantified using a luminometer (Berthold Lumat, 9501) and standardized according to β-galactosidase activity. Relative Luciferase units normalized for β-galactosidase activity (Relative Luciferase Unit; RLU) were determined and plotted as an

average of triplicate determinations and error bars represent standard deviations of the triplicate values.

Co-immunoprecipitation analysis An equal amount of total cellular protein (250μg) was diluted with NP-40 lysis buffer to a final concentration of 1 μg/μl. Protein complexes were immunoprecipitated with the indicated antibody and 25μl of protein G-Plus Sepharose beads (50% slurry) (Santa Cruz Biotechnology) by incubation at 4°C overnight on a rotating platform. The beads were washed with three changes of NETN wash buffer (0.1% NP-40, 150mM NaCl, 1mM EDTA, and 50 mM Tris-HCl pH 8.0). Beads were boiled in SDS sample buffer, and protein complexes were resolved by SDS-PAGE and immuno-blotted as described above.

Results

Constitutive nuclear localization of Smad7 bypasses its inhibitory role at the TGFβ receptor complex. Since our preliminary studies suggested a possible dual role for Smad7 at the level of the TGFβ receptor and also in the nucleus, we therefore aimed to dissect these potentially independent facets of Smad7 activity in a systematic manner. Initially, we sought to engineer a nuclear localized Smad7 that was independent of receptor mediated events in order to test whether this property of Smad7 could recapitulate the pro-myogenic effect that we have previously documented for the wild-type Smad7 molecule (26). One strategy was to add a nuclear localization signal (NLS) to Smad7. The rationale being that such a modification of Smad7 would abrogate its ability

to interfere with R-Smad activation by the receptor while concomitantly localizing it to the nuclear compartment where, based on our previous observations, we predicted it might still function in the control of myogenic gene expression.

Therefore, we fused the NLS of SV40 at the C-terminus of Smad7. In addition, to track the sub-cellular localization of the Smad7-NLS fusion protein in living cells, we also generated a fusion in which we added EGFP at the N-terminus of Smad7 (figure 1A). The fusion proteins were expressed in transfected C2C12 cells at the expected molecular weights. Addition of the NLS to Smad7 has no apparent effect on the expression levels of the Smad7 proteins (whether conjugated with EGFP or not) in myogenic cells (figure 1B). In C2C12 cells, exogenously expressed EGFP-Smad7 localized to the nucleus and cytoplasm, while EGFP-Smad7-NLS was localized almost exclusively to the nucleus as indicated by EGFP signal localization (figure 1C). To quantitate these observations, we randomly chose 10 fields and scored sub-cellular localization of the EGFP signals. The results, summarized in figure 1D, document that, in contrast to EGFP-Smad7, EGFP-Smad7-NLS essentially localizes to the nucleus. We further determined the sub-cellular localization of the Smad7 fusion proteins by biochemical fractionation of cytoplasmic and nuclear extracts. Western blotting analysis of the fractionated samples for positive markers of the cytoplasmic (MEK1/2) and nuclear fractions (c-Jun) revealed a very good level of enrichment in the respective fractions (figure 1E). Subsequent analysis of the different Smad7 proteins showed that EGFP-Smad7 was detected in both the nuclear and cytoplasmic fractions whereas, as predicted, EGFP-Smad7-NLS was essentially localized in the nuclear fraction as assessed by three different primary antibodies (recognizing myc, Smad7, and GFP proteins in figures 1E and 1F). Thus, we concluded that adding the NLS

to Smad7 effects a very efficient re-localization of Smad7 in the nucleus and largely eliminates the cytoplasmic accumulation which is characteristic of the wild type protein. Thus, based on the biochemical and fluorescence data we concluded that we have effectively engineered a variant of SMAD7 that is localized in the nucleus and should be incapable of functioning at the level of the TGFβ receptor.

We next assessed whether the engineered changes in localization of Smad7 resulted in functional alterations in its properties. Firstly, TGFβ potently activated a TGFβ/Smad3 dependent reporter gene (3TP-lux) activity, and TGFβ induced 3TP-lux activity was reduced in the presence of EGFP-Smad7 (figure 1G), consistent with the known function of wild-type Smad7. However, as we predicted, ectopic expression of EGFP-Smad7-NLS did not interfere with 3TP-lux activation by TGFβ as would be seen by wild type Smad7 (figure 1G). However, we noted that 3TP-lux consists of a Smad Binding Element (SBE) and also 3 copies of the TPA Responsive Element (TRE), which recognizes activator protein-1 (AP-1) transcription factor. Because we have recently observed that TGFβ can activate AP-1 in this system (data not shown), we also constructed a multimerized Smad3 binding site (CAGA) driven luciferase reporter gene ((CAGA)X10-luc) to test whether this effect was primarily dependent on the SBE and independent of the TREs in 3TP Lux (11). In agreement with the results observed for 3TP-lux, Smad7 inhibited TGFβ induced (CAGA)X10-luc activity, while Smad7-NLS expression had essentially no effect on reporter gene activation by TGFβ (figure 1H). These results indicate that constitutive Smad7 localization to the nucleus bypasses repression of TGFβ signaling at the level of the receptor. Thus, the cytoplasmic localization of Smad7 is required for Smad7's 'canonical' inhibitory effect on the TGFβ/Smad3 pathway.

Nuclear Smad7 enhances MyoD's transcriptional activation properties independent of inhibiting the TGFβ/Smad3 pathway. Having successfully engineered Smad7-NLS, which is localized to the nucleus and is unable to interfere with TGFβ receptor mediated Smad3 activation, we tested whether this molecule could still function to enhance myogenesis. Since we previously observed that Smad7 can physically associate with MyoD and enhances MyoD's transcriptional properties (26), we sought to determine whether Smad7-NLS retains the ability to potentiate MyoD's transcriptional properties. First, we determined the effect of exogenous expression of Smad7 on nuclear MyoD protein levels with or without addition of TGFβ (1ng/ml). Exogenously expressed Smad7 and Smad7-NLS were detected in the nuclei, and in agreement with the above results, Smad7-NLS accumulated in the nucleus in considerably larger amounts than that of wild-type Smad7 (figure 2A). Although it was previously reported that MyoD protein levels are down-regulated when cells are exposed to TGFβ, there was no effect of exogenous Smad7 on endogenous MyoD protein levels in the presence or absence of TGFβ. In addition, ectopic expression of Smad7-myc and Smad7-NLS-myc had no effect on endogenous expression of MyoD, Myf5, and c-Jun, and transfection efficiency was not affected by the expression of Smad7 fusion proteins as assessed by co-transfected dsRed2 expression (figure 2B).

To further examine whether Smad7 enhances myogenesis independent of Smad7's 'canonical function' of inhibiting R-Smad activation mediated by the activated TGFβ receptor complex, we silenced TGFβ and Myostatin signaling by expression of a dominant negative (DN) form of the corresponding receptors (figure 2C). Activity of a

Smad3 dependent reporter gene (3TP-luc) was repressed by expressing the DN form of activin type II receptor (DN-ActIIR), of TGFβ type II receptor (DN-TbIIR), or a combination of both. Under these conditions of complete receptor blockade, Smad7 still activated MyoG promoter-reporter gene activity suggesting that Smad7 enhances MyoD's transcriptional properties in a manner independent of TGFβ-Smad3 signaling.

Nuclear Smad7 enhances muscle differentiation. Based on the above observations on the *myog* promoter, we next tested the hypothesis that the pro-myogenic role of Smad7 could be recapitulated by the nuclear localized Smad7. To address this question, we first examined the effect of Smad7-NLS and Smad7 on MyoD's transcriptional activity. As indicated by a *myog* promoter-luciferase reporter gene (pMyoG-luc), Smad7-NLS enhanced MyoD driven *myog* reporter gene activity to a similar extent to that of wild-type Smad7 (figure 3A) without affecting MyoD protein levels (figure 3B). Next, Smad7-NLS was expressed in C2C12 myoblasts in which a muscle differentiation marker gene, *muscle creatine kinase* (*mck*) promoter EGFP reporter construct (pMCK-EGFP) was included to monitor myogenesis. Transfected cells were marked by co-transfection of pCMV-dsRed2. Progression of muscle differentiation was monitored by documenting EGFP expression driven by the *mck* promoter. After 48hrs in differentiation conditions, the cells transfected with empty expression vector (pcDNA3) started forming multinucleated myotubes, and *mck* driven EGFP signals were observed in these myotubes (figure 3C). In agreement with our previous studies (26), Smad7 transfected cells generated larger caliber myotubes and stronger MCK-EGFP signals compared to those in the control cells. Moreover, Smad7-NLS expressing cells

showed a similar enhancement of myogenesis to that observed with wild-type Smad7, and these observed effects were restricted to transfected cells (dsRed2 positive cells) with Smad7 or Smad7-NLS expression in contrast to empty vector transfected cells (figure 3C).

Therefore, the Smad7-NLS chimera preserves the nuclear function of Smad7 such that it enhances MyoD's transactivation properties and promotes myogenesis whilst completely losing its capacity to inhibit the TGFβ-Smad signaling pathway. These results indicate that Smad7 nuclear localization, independent of its ability to inhibit receptor regulated Smad activation, is sufficient for Smad7's pro-myogenic effect.

The C-terminus (amino acids 409 to 426) of Smad7 is required for nuclear accumulation and pro-myogenic activity. We previously documented that Smad7 promoted myogenesis and potently inhibited TGFβ induced Smad3 activity in C2C12 cells (26) (figure 1G and H and figure 3D). In contrast, Smad7T, a deletion mutation which lacks the last 18 amino acids (aa) (Δ409-426) (figure 4A), was not able to repress activation of Smad3 by TGFβ (figure 4B left). In addition, we observed that Smad7T failed to reverse the inhibition of myogenesis by Myostatin (26), and was incapable of enhancing activity of myogenic reporter genes such as the *myog* gene (figure 4B right). Next, we assessed the possibility that Smad7T might be dysfunctional because of its localization in the cell. To investigate this, expression vectors for EGFP-Smad7 and EGFP-Smad7T were generated (figure 4A) and their sub-cellular location was documented (figure 4C and D). We found that EGFP-Smad7 localized to both nucleus and cytoplasm in C2C12 cells as previously documented. However, EGFP-Smad7T was

essentially excluded from the nucleus (figure 4C and D) indicating that the 18 amino acids at the C-terminus of Smad7 are required for its proper nuclear localization (20) and, importantly, nuclear accumulation is required for the pro-myogenic function of Smad7. This observation lends further support to our idea that the pro-myogenic role of Smad7 resides in its ability to function in the nucleus although Smad7T does also seem to be deficient in its ability to abrogate canonical TGFβ signaling (figure 4B).

Smad7 can promote muscle differentiation by antagonizing the inhibitory effect of activated MEK on MyoD's transcriptional activation properties. We previously demonstrated that Smad7 physically interacts with MyoD and reduction of Smad7 expression by siRNA technology severely represses myogenesis suggesting that co-operation between MyoD and Smad7 is required for myogenesis (26). We, and others, reported that the activity of MyoD is repressed by its interaction with MEK (39, 47). The MEK-MyoD interaction may be a nodal point for myogenic repression since a number of cytokines such as Cardiotrophin-1 (CT-1) inhibit myogenesis by promoting the association of MyoD with MEK (39). Based on these observations, we postulated that one nuclear function of Smad7 might be to antagonize the repressive function of MEK on MyoD's transactivation properties.

To address this, we utilized the pMyoG-luc reporter gene to quantify MyoD's transcriptional activity in response to perturbations in MEK and Smad7 activity. Exogenously expressed, activated MEK1 (Act.MEK1) repressed MyoD driven pMyoG reporter gene activity in a dose dependent manner as previously reported (47) without affecting protein levels of Smad7 and MyoD (figure 5A and B). Also, Smad7 partially

reversed the inhibitory effect of Act.MEK1 on MyoD (figure 5A) suggesting that Smad7 and MEK signaling converge on MyoD in a reciprocal manner to regulate muscle differentiation. We tested this idea further in a myogenic conversion assay which takes advantage of MyoD's capacity to induce myogenesis in the 10T1/2 fibroblast cell line. The results of these studies were striking in that ectopic expression of an active form of MEK1 prevents induction of MyoG by MyoD in this assay (figure 5C) and Smad7 and Smad7-NLS enhance MyoD driven myogenesis (figure 5D). However, ectopic expression of Smad7 as well as Smad7-NLS reverses this antagonism of MyoG induction by MEK1 (figure 5E, and F). It is worth mentioning that these reciprocal effects of Smad7 and MEK1 on target genes expressed in muscle appear to be dependent on MyoD since another MEK inducible gene, *sprr1a* (48) which is not targeted by MyoD but is expressed in muscle cells, was not repressed by exogenous expression of Smad7 (data not shown). We further observed precocious up-regulation of MyoG, which is a key target of MyoD, by ectopic expression of Smad7-myc and Smad7-NLS-myc in differentiating myoblasts (figure 5G).

To further characterize the MyoD-Smad7 interaction, we investigated the transcriptional activity of a number of MyoD deletion mutants (figure 6A and B) in the presence of either Act.MEK1 or Smad7. Western blotting analysis showed that MyoD or its deletion mutants are appropriately expressed in transfected cells (figure 6B). As previously reported (47), Act.MEK1 repressed pMyoG-reporter gene activity driven by MyoD or a series of deletion mutants except for one lacking aa 3-56 (MyoDΔ3-56) (figure 6C). Cardiotrophin-1 (CT-1), which inhibits myogenesis by activation of the MEK/ERK pathway (39), also repressed this reporter gene activity (figure 6D). Smad7 enhanced

MyoD's transcriptional activity, but this enhancement was diminished by deletion of the N-terminal part of MyoD (aa 3-56 or aa 63-99) (figure 6E). These data indicate that MEK1 and Smad7 may functionally interact with an overlapping region of MyoD suggesting that their interaction might be mutually exclusive. To explore this possibility, we exogenously expressed MyoD and act.MEK1 with or without a myc epitope tagged Smad7 to assess the amount of MEK1 in the MyoD containing immuno-complex. We postulated that if MEK1 and Smad7 associate with MyoD protein in a competitive manner, the amount of MEK1 interacting with MyoD should be reduced in the presence of Smad7. Smad7 and MEK1 were detected in the immuno-complex precipitated by a MyoD antibody (figure 6F). However, in the presence of enhanced Smad7 expression, there was no apparent effect on the amount of MEK1 in the MyoD immuno-complex suggesting that Smad7 and MEK1 associate with the MyoD protein in a non-competitive manner.

Smad7 reverses inhibition of muscle differentiation by activated MEK by CT-1.

Recently, we identified that CT-1 is a physiological regulator of skeletal muscle differentiation, and CT-1 inhibits muscle differentiation through activation of MEK which antagonizes MyoD activation (39). Therefore, we used CT-1 as a biological regulator of muscle differentiation and as an activator of MEK for these experiments. In agreement with the above, CT-1 inhibited *myog* promoter-reporter gene activation by MyoD in a dose dependent manner, and exogenously expressed Smad7 reversed CT-1's inhibitory effect on the *myog* promoter (figure 7A). Collectively, these results suggest that activation of the MEK/ERK pathway prevents premature differentiation of myoblasts

by repressing MyoD's transcriptional activation properties (39). Moreover, Smad7 antagonizes MEK's inhibitory effect on MyoD to induce transcription of the *myog* gene (figure 5A), which is an essential step for myogenesis. Because of these observations, we hypothesized that altering the levels of Smad7 may cause precocious differentiation even in the presence of CT-1. We previously reported that exogenously expressed Smad7 accelerated formation of myotubes and up-regulated pMCK-EGFP activity compared to those of control cells (pcDNA3) (26) (figure 3C and 7B). As previously observed (39), CT-1 treated C2C12 cells in DM were repressed from differentiation as indicated by prevalence of the mononucleated myoblast phenotype and also the lack of pMyoG-luc activation after 48hrs in DM. Importantly, these inhibitory effects of CT-1 on myogenesis were essentially reversed by exogenous expression of Smad7 (figure 7B), suggesting that the balance between activated MEK and Smad7 regulates the initiation of muscle differentiation (figure 8).

Discussion

In this study, we document that nuclear Smad7 enhances MyoD's transcriptional activation properties. This is the first report to identify a clear biological function of nuclear Smad7. We found that Smad7 in the nucleus interacts with MyoD and enhances MyoD's ability to induce muscle differentiation. Our previous study showed that reduced expression of Smad7 by siRNA technology antagonized myogenic differentiation suggesting that Smad7 is an essential component of the program of myogenic differentiation (26). It is well established that MyoD requires activation to induce

myogenesis since myogenic lineage determining factors, MyoD and Myf5, are detected in undifferentiated myoblasts where they are inactive as initiators of the myogenic gene expression program. The exact molecular mechanism by which the MRFs shift from a repressed to an active state to induce the myogenic program of gene expression is not completely understood although a number of studies to date have found that MyoD activity is extensively regulated by protein-protein interactions. Data presented here implicate Smad7 as an essential co-factor in the myogenic cascade. Independent of the well known function of Smad7 in the cytoplasm, nuclear Smad7 physically interacts with MyoD and potentiates MyoD's transcriptional activation properties.

Since MRF activity is sensitive to extracellular secreted factors, it is reasonable that downstream signaling molecules associated with these signaling pathways will play an important role for the regulation of MRF activity. Accumulating evidence indicates that the MEK-extracellular regulated kinase (ERK) pathway also plays a fundamental role on MyoD's transcriptional activation properties. Interestingly, a previous report showed that activated MEK physically interacts with MyoD and inhibits MyoD activity (47). In addition, we have recently documented that CT-1 represses myogenesis through inactivation of MyoD by activation of the MEK/ERK pathway (39). In this study, we demonstrate that Smad7 can reverse CT-1's inhibitory effect on myogenesis. This effect is not confined to CT-1 since Smad7 is also capable of reversing the inhibitory effect of MEK on MyoD. Since MEK and Smad7 can form complexes with MyoD, MEK signaling and Smad7 converge on MyoD. Interestingly, Smad7 expression is regulated by MyoD at the transcriptional level through the E-box in the *smad7* promoter. Therefore, Smad7 and MyoD may constitute a mutually reinforcing feed-forward loop to initiate

myogenesis. Thus, the down-regulation of *smad7* mediated by MEK activation could be a common mechanism to inhibit myogenesis utilized by a number of different growth factors and cytokines such as CT-1, FGF, EGF and PDGF (figure 7). Interestingly, although Myostatin, a member of the TGFβ family, and CT-1 mediated repression of myogenesis were 'rescued' by exogenous Smad7 expression, Smad7 could not reverse TGFβ's inhibitory phenotype (26). However, TGFβ's inhibitory effect is partially reversed by MEK inhibitors (T.Miyake, unpublished observation). Therefore, activation of MyoD's transcriptional activation properties either by up-regulation of an activator, for example Smad7, or down-regulation of an inhibitor, for example active MEK, may constitute a key nodal point for the regulation of MyoD activity and subsequently the decision to initiate differentiation.

Interestingly, Levy and Hill identified Smad4 dependent and independent TGFβ target genes by reducing Smad4 expression by siRNA technology (29) indicating that there are divergent groups of TGFβ responsive target genes regulated by different facets of the signaling pathway. Smad7-NLS could also prove to be a useful tool in dissecting non-canonical aspects of TGFβ signaling pathway. A similar approach of dissecting the TGFβ independent target genes of the nuclear form of Smad7 has the potential to identify a unique group of myogenic genes that are regulated by MyoD and Smad7.

In summary, we document a novel function of nuclear Smad7 in myogenic cells (figure 7). Nuclear Smad7 antagonizes the inhibitory effect of MEK on MyoD's transcriptional activation properties and, importantly, enhances myogenesis independent of its inhibitory role in TGFβ-Smad3 signaling. These observations document a novel 'non-canonical'

nuclear role for Smad7 in modulating the properties of MyoD and potentiating myogenic differentiation.

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Figure Legends

Figure 1. **Constitutive nuclear localization of Smad7 bypasses its inhibitory role at the TGFβ receptor complex** A) A schematic presentation of Smad7 proteins. NLS; SV40 large-T nuclear Localization Sequence, EGFP; enhanced green fluorescence protein. (black box; NLS). B) C2C12 cells were transfected with indicated constructs with pCMV-dsRed2, and expression was verified by Western blotting analysis with indicated antibodies. dsRed2 blot was included as an indicator of transfection efficiency. C) C2C12 cells were transfected with indicated EGFP constructs (1μg) for expression of EGFP-fusion proteins. The transfected cells were maintained in DM for 16hrs. The cell morphology was recorded by phase-contrast microscopy. Sub-cellular localization of EGFP, EGFP-Smad7, or EGFP-Smad7-NLS was monitored by the green fluorescence protein signal. Overlay images were generated from the phase contrast and EGFP micrograph. D) Five fields were randomly chosen in each condition, and sub-cellular localization of the indicated EGFP proteins were scored as 'whole', 'nucleus' or 'cytoplasm'. Graphs were generated indicating the percentage of the sub-cellular localization of EGFP signal over total EGFP positive cells. E and F) C2C12 cells were transfected with the indicated construct, and subjected to extraction of nuclear/cytoplasmic proteins using NE-PER®. Nuclear and cytoplasmic protein samples were analyzed by Western blotting techniques. MEK1/2 and c-Jun were markers for cytoplasmic and nuclear proteins respectively. G) C2C12 cells were transfected with 3TP-luciferase reporter gene construct (3TP-Lux) (0.5μg), and expression vector of EGFP, EGFP-Smad7, or EGFP-Smad7-NLS (1μg). In addition, to monitor transfection efficiency, pCMV-β-gal construct (0.3μg) was included in each condition. The

transfected cells were maintained for 16 hrs in TGFβ (1ng/ml) or its solvent in DM. Total protein samples were harvested with a luciferase lysis buffer. Luciferase activity in each condition was measured independently and normalized according to β-Galactosidase activity: relative luciferase unit (RLU). The bar represents the average of the RLU of the three individually transfected samples (+/- STD). H) C2C12 cells were transfected with pGL3-basic (pGL3) or (CAGA)X10-Luciferase reporter gene construct (0.5μg), and expression vector for Smad7, or Smad7-NLS, or an empty expression vector (pcDNA3)(1.0μg) as a control. In addition, to monitor transfection efficiency, pCMV-β-gal construct (0.3μg) was included in each condition. The transfected cells were maintained for 16 hrs in TGFβ (1ng/ml) or its solvent in DM. Total protein samples were analyzed in the same way as stated above. Each bar represents the mean of triplicate sample (+/- SD).

Figure 2. **Nuclear Smad7 enhances MyoD's transcriptional activation properties independent of inhibiting the TGFβ/Smad3 pathway** A) C2C12 cells were transfected with expression vectors for Smad7-myc, Smad7-NLS-myc, or control (pcDNA3) (1.0μg). The transfected cells were maintained for 16 hrs in TGFβ (1ng/ml) or its solvent in DM. Nuclear protein was extracted by using NE-PER®. The amount of indicated nuclear protein was visualized with standard Western blotting technique. Equal protein loading was monitored by c-Jun immunoblot. Nuclear Smad7 with a myc-epitope tag was identified by anti-myc antibody. B) C2C12 cells were transfected with the indicated constructs. Expression levels of the indicated proteins were assessed by Western blotting analysis. An Actin blot indicated equal loading of the protein samples,

and a dsRed2 blot showed that similar transfection efficiency. C) C2C12 cells were transfected with 3TP-Luc, pMyoG-Luc or pGL3-basic reporter gene construct (0.3μg). In addition, expression vectors for a dominant negative form of type IIB Activin receptor (DN-ActII), dominant negative typeII TGFβ receptor (DN-TbIIR), or a combination of both (1.0μg each, total 2.0μg). Expression vector either for Smad7 or empty control (pcDNA3) (1.0μg) were added to each condition. pCMV-β-gal construct (0.3μg) was included in each condition to monitor transfection efficiency. The transfected cells were maintained for 16 hrs in DM. Total protein samples were analyzed as the same way as stated above to calculate each RLU. Fold-activation was calculated with respect to the control. The bar represents the average of the fold-activation of the three individually transfected samples (+/- SD).

Figure 3. **Nuclear Smad7 enhances muscle differentiation** A) C2C12 cells were transfected with a pMyoG-Luciferase reporter gene construct (pMyoG-luc) (0.5μg) with MyoD expression vector (pEMSV-MyoD) (1.0μg). In addition, expression vector for Smad7, or Smad7-NLS, or an empty expression vector (pcDNA3) (1.0μg) as a control was included. pCMV-β-gal construct (0.3μg) was included in each condition to monitor transfection efficiency. The transfected cells were maintained for 16 hrs in DM. The bar represents the average of the fold-activation of the three individually transfected samples (+/- STD). B) C2C12 cells were transfected with indicated constructs, and the transfected cells were subjected to Western blotting analysis for the indicated protein expression. C) C2C12 cells were plated at equal density and transfected with pCMV-dsRed2 (0.5μg) and pMCK-EGFP constructs (1.0μg). The transfected cells were maintained in DM for 48 hrs

to induce myotube formation. The cell morphology was recorded by phase-contrast microscopy and transfected cells were monitored by the dsRed2 signal. MCK promoter activity was assessed by the EGFP signal. Overlay images were generated from the phase contrast and EGFP micrograph.

Figure 4. **The C-terminus (amino acids 409 to 426) of Smad7 is required for nuclear accumulation and pro-myogenic activity** A) A schematic presentation of Smad7 proteins. Smad7T; TGFβ receptor interaction region was deleted from Smad7. B) C2C12 cells were transfected with 3TP-Lux (left panel) or, pMyoG-Luc (right panel) (0.5μg) and expression vector of Smad7, Smad7T, or empty control (pcDNA3) (1.0μg). MyoD expression vector (pEMSV-MyoD) or empty vector (pEMSV) (1.0μg) was also included (right panel). In addition, to monitor transfection efficiency, pCMV-β-gal construct (0.3μg) was included in each condition. The transfected cells were maintained for 16 hrs in TGFβ (1ng/ml) or its solvent in DM (left panel), or in DM (right panel). Luciferase activity in each condition was measured independently and normalized according to β-galactosidase activity: relative luciferase unit (RLU). The bar represents the average of the RLU of the three individually transfected cellular samples (+/- STD). C) C2C12 cells were transfected with indicated EGFP constructs (1.0μg) for expression EGFP-fusion peptides. The cell morphology was recorded by phase-contrast microscopy. Sub-cellular localization of EGFP-Smad7, or EGFP-Smad7T was monitored by the EGFP signal. D) Five fields were randomly chosen in each condition, and sub-cellular localization of the indicated EGFP proteins were scored as 'whole', 'nucleus' or 'cytoplasm'. Graphs were

generated percentage of the sub-cellular localization of EGFP signal over total EGFP positive cells.

Figure 5. **Smad7 can promote muscle differentiation by antagonizing the inhibitory effect of activated MEK on MyoD's transcriptional properties** A) C2C12 cells were transfected with a pMyoG-Luc (0.5μg), a MyoD expression vector (1.0μg), a pCMV-β-gal (0.3μg), and also increasing amounts (0, 0.1, 0.4, 0.8, and 1.0μg from left) of expression vector for the activated form of MEK1 (Act.MEK1) with a combination of the empty vector for control (3.0, 2.9, 2.6, 2.2 and 2.0μg) (total 3.0μg for each condition). In addition, the expression vector for Smad7 (1.0μg) or empty control was added. The transfected cells were maintained in DM for 16hrs. The cells were harvested and subjected to Luciferase assay and β-gal assay. Luciferase activity was normalized according to the β-galactosidase activity to calculate the RLU (+/- STD). B) C2C12 cells were transfected with the indicated constructs (5μg of Smad7-myc and MyoD expression vectors in all conditions with increasing amount (0, 0.5, 2, 2.5, and 5μg) of Act.MEK1. The transfected cells were maintained in DM for 16hrs. Expression levels of the indicated proteins were assessed by Western blotting analysis. An Actin blot indicated equal loading of the protein samples. C) C3H10T1/2 cells were transfected with the indicated constructs (5μg of MyoD expression vector in all conditions with +/- myc-Smad7 (5μg), and increasing amount (0, 0.5, 1, and 5μg) of Act.MEK1. The transfected cells were maintained in DM for 16hrs. Expression levels of the indicated proteins were assessed by Western blotting analysis. Endogenous MyoG and c-Jun protein levels were also assessed. An Actin blot indicated equal loading of the protein samples. D) C3H10T1/2 cells were

transfected with Smad7-NLS-myc, Smad7-myc, or control empty vector (8μg) and constant amount of MyoD expression vector (5μg). The transfected cells were maintained in DM for 16hrs to induce MyoG induction and subjected to extraction of nuclear/cytoplasmic proteins using NE-PER®. Nuclear and cytoplasmic protein samples were analyzed by Western blotting techniques. Endogenous MEK1/2 and c-Jun were markers for cytoplasmic (C) and nuclear (N) proteins respectively. Expression levels of the indicated proteins in each cell compartment were assessed by Western blotting analysis. For the MyoD blot the arrow indicates MyoD protein and * indicates a non-specific cytoplasmic cross reactant. E and F) C3H10T1/2 cells were transfected Smad7-myc (E), Smad7-NLS-myc (F), or control empty vector (0, 2, or 8μg) and Act.MEK1 (0, 2 or 8μg) with constant amount of MyoD (4μg) expression vectors. Total protein samples were extracted and analyzed by Western blotting techniques. Endogenous MEK1/2 and c-Jun were markers for cytoplasmic and nuclear proteins respectively. Expression levels of the indicated proteins were analyzed by Western blotting technique. G) C2C12 cells were transfected with Smad7-myc, Smad7-NLS-myc, or empty vector using lipofectamine (Invitrogene). The transfected Cells were maintained in DM for 48 h after transfection to induce differentiation and total protein was extracted and expression levels of the indicated proteins were analyzed by Western blotting. Actin was used as a loading control and dsRed2 as a marker for transfection efficiency.

Figure 6. **Smad7 and MEK1 interact with MyoD** A) A schematic presentation of MyoD protein. The number corresponds to amino acid co-ordinates. B) Western blotting analysis of expression of deletion mutant of MyoD proteins. C3H10T1/2 cells were

transfected with indicated expression vector (1.0μg) for the expression of indicated MyoD deletion mutant, wild-type MyoD protein, or empty control. The transfected cells were maintained in DM for 16hrs and harvested for total protein samples. The extracted total proteins were subjected to Western blotting analysis described in the *Experimental Procedure* to confirm expression of corresponding MyoD protein. C-E) C3H10T1/2 cells were transfected with pMyoG-Luc (0.5μg), and indicated MyoD wild-type, or deletion mutant forms of MyoD, or empty expression vector (1.0μg), and a pCMV-β-gal (0.3μg). In addition, expression vector (1.0μg) for active form of MEK1 or empty control (C), or Smad7 or empty control (E) was included. The transfected cells were maintained in DM for 16hrs for D&F or DM containing CT-1 (10ng/ml) or solvent (D). The cells were harvested and subjected to Luciferase assay and β-gal assay. Luciferase activity was normalized according to the β-galactosidase activity to calculate the RLU (+/-STD). F) C3H10T1/2 cells were transfected with combinations of the indicated constructs. Total protein samples were extracted from the cells maintained in DM. Exogenous-expression of MyoD, an activated form of MEK1, or Smad7-myc was detected by immuno-blotting (IB) (10μg loading) with the specific antibodies. A co-immuno-precipitation (Co-IP) analysis was performed with the total protein extract (250μg) with MyoD antibody (mouse) and proteinG conjugated beads. Precipitated immuno-complexes were eluted off the proteinG beads and subjected to immunoblotting with MEK antibody or myc antibody.

Figure 7. **Smad7 reverses inhibition of muscle differentiation by activated MEK by CT-1** A) C2C12 cells were transfected with a pMyoG-Luc (0.5μg) or pGL3-basic

luciferase reporter gene construct with MyoD expression vector or empty vector (1.0μg) and Smad7 expression vector or pcDNA3(1.0μg). pCMV-β-gal (0.3μg) was also added in each condition for monitoring transfection efficiency. The transfected cells were maintained in DM containing either CT-1 (3ng/ml or 10ng/ml) or solvent for 16hrs. The cells were harvested and subjected to Luciferase assay and β-gal assay. Luciferase activity was normalized according to the β-galactosidase activity to calculate the RLU. The bar represents the average of the RLU of the three individually transfected samples (+/- SD). B) C2C12 cells were plated at equal density and transfected with pCMV-deRed2 (0.5μg) and pMCK-EGFP constructs (1.0μg) , and either the expression vector for Smad7 or empty control (pcDNA3) (1.0μg). The transfected cells were maintained in CT-1 (10ng/ml) or solvent containing DM for 48 hrs to induce myotube formation. The cell morphology was recorded by phase-contrast microscopy and transfected cells were monitored by the red fluorescence signal. MCK promoter activity was assessed by the green fluorescence signal.

Figure 8. **A model of nuclear Smad7 function in myogenesis** The depicted schematic indicates the hierarchal relationship between MyoD, MEK, and Smad7 in the control of muscle specific genes based on data presented here.

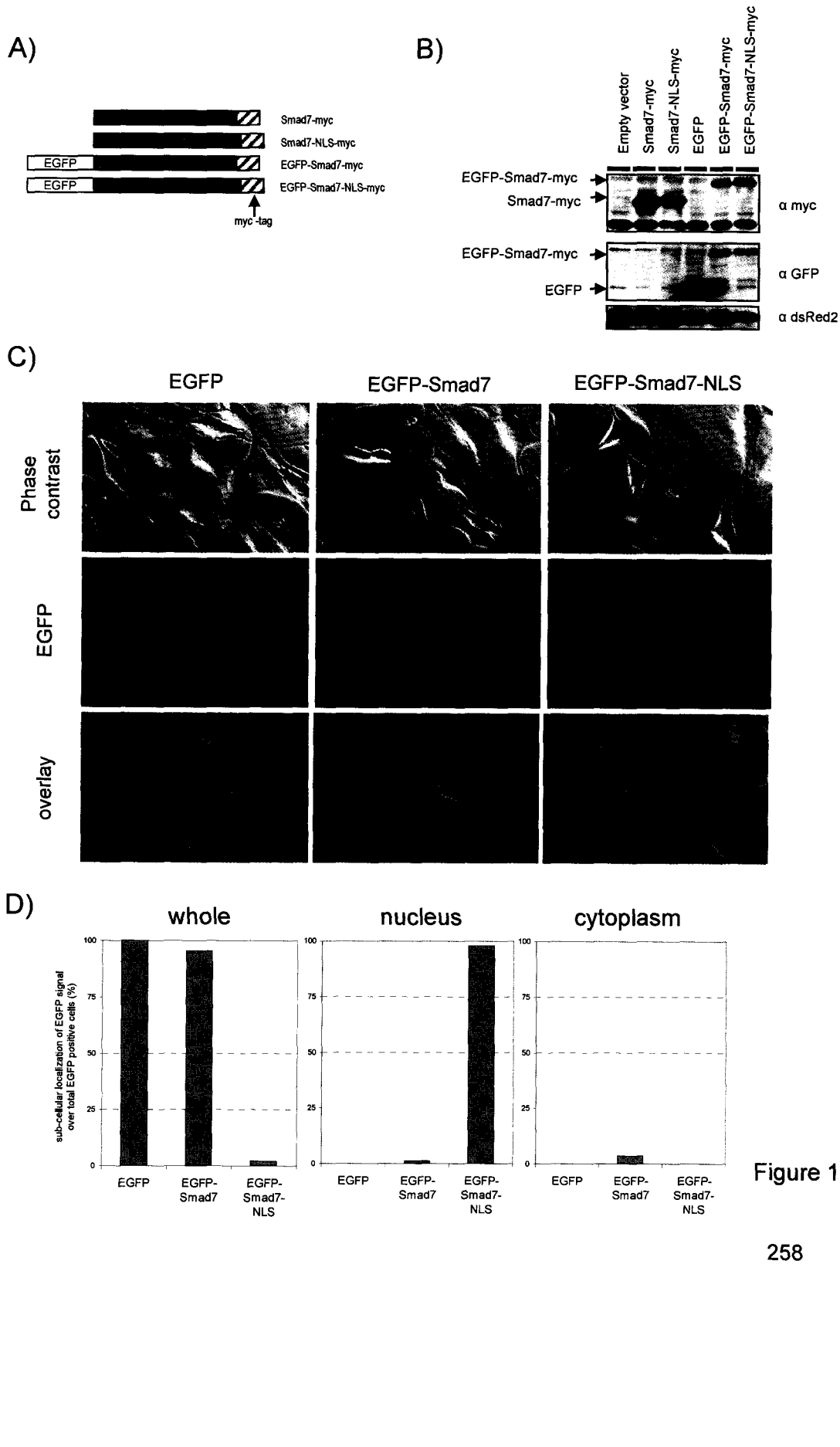

A)
Smad7-myc
Smad7-NLS-myc
EGFP
EGFP-Smad7-myc
EGFP
EGFP-Smad7-NLS-myc
myc -tag
B)
Empty vector
Smad7-myc
Smad7-NLS-myc
EGFP
EGFP-Smad7-myc
EGFP-Smad7-NLS-myc
EGFP-Smad7-myc
Smad7-myc
α myc
EGFP-Smad7-myc
EGFP
α GFP
α dsRed2
C)
EGFP
EGFP-Smad7
EGFP-Smad7-NLS
Phase contrast
EGFP
overlay
D)
whole
nucleus
cytoplasm
sub-cellular localization of EGFP signal over total EGFP positive cells (%)
100
75
50
25
0
EGFP
EGFP-Smad7
EGFP-Smad7-NLS

Figure 1

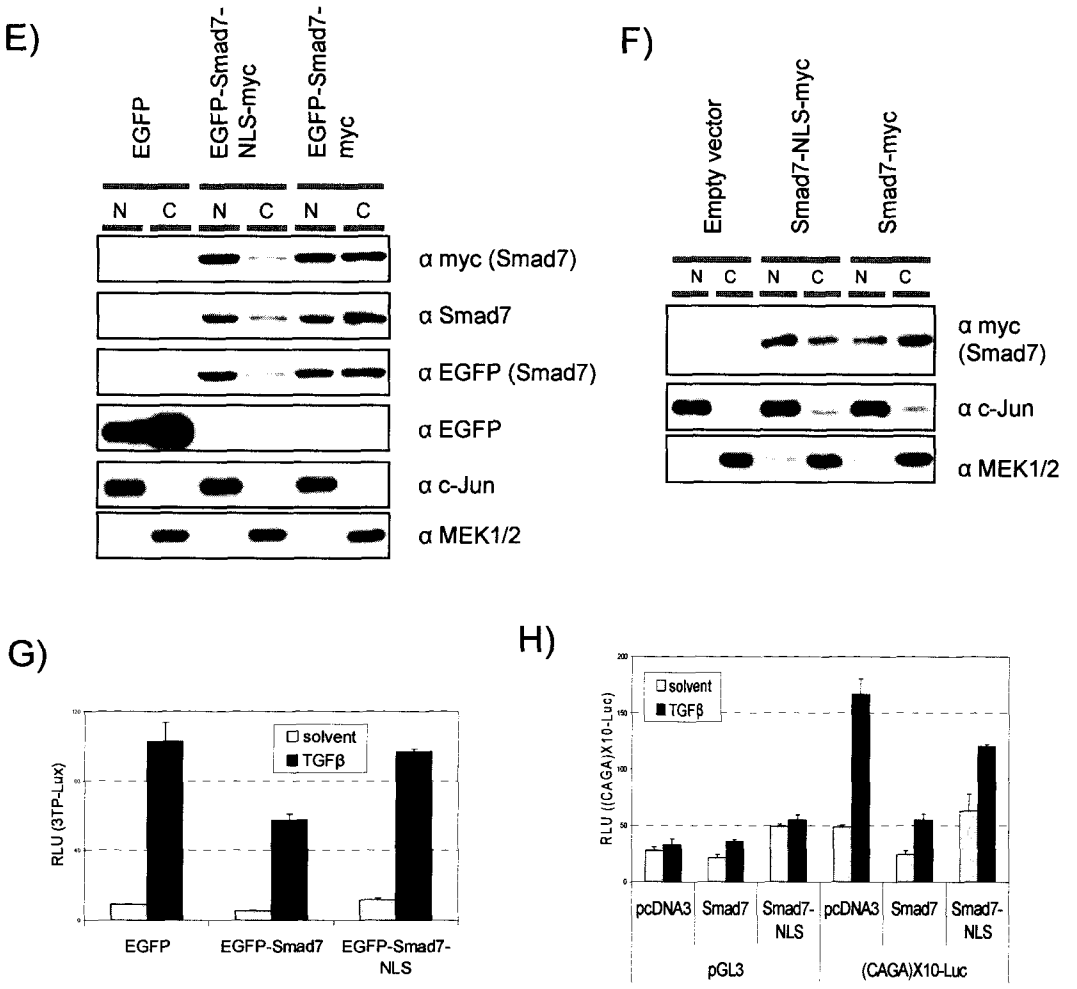
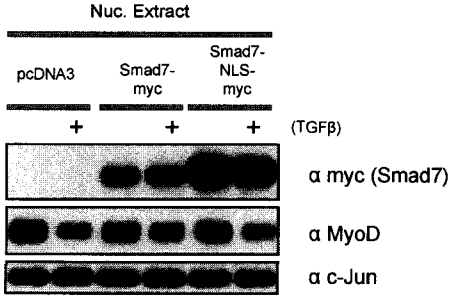
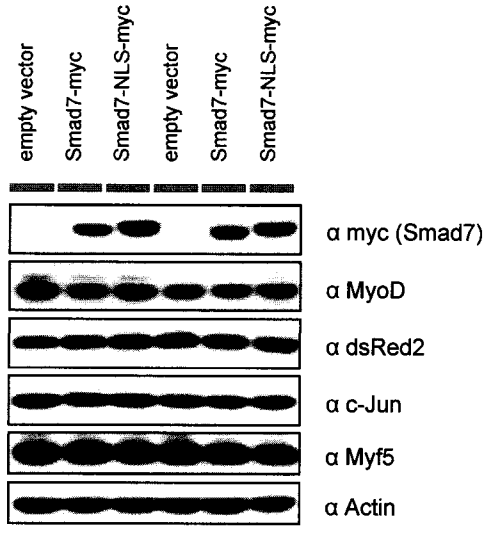

E)
EGFP
EGFP-Smad7-NLS-myc
EGFP-Smad7-myc
N C N C N C
α myc (Smad7)
α Smad7
α EGFP (Smad7)
α EGFP
α c-Jun
α MEK1/2
F)
Empty vector
Smad7-NLS-myc
Smad7-myc
N C N C N C
α myc (Smad7)
α c-Jun
α MEK1/2
G)
RLU (3TP-Lux)
solvent
TGFβ
EGFP
EGFP-Smad7
EGFP-Smad7-NLS
H)
RLU ((CAGA)X10-Luc)
solvent
TGFβ
pcDNA3
Smad7
Smad7-NLS
pcDNA3
Smad7
Smad7-NLS
pGL3
(CAGA)X10-Luc

Figure 1

A)


Nuc. Extract
pcDNA3
Smad7-myc
Smad7-NLS-myc
+ + + (TGFβ)
α myc (Smad7)
α MyoD
α c-Jun

B)


empty vector
Smad7-myc
Smad7-NLS-myc
empty vector
Smad7-myc
Smad7-NLS-myc
α myc (Smad7)
α MyoD
α dsRed2
α c-Jun
α Myf5
α Actin

C)

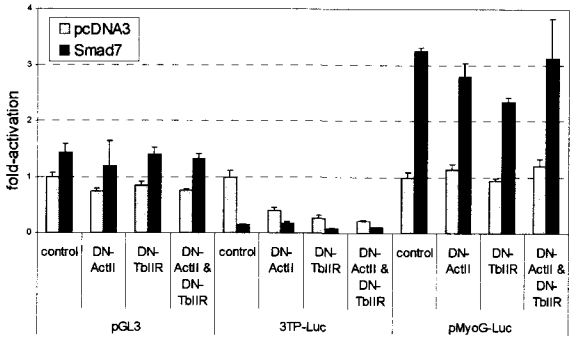

pcDNA3
Smad7
fold-activation
control
DN-ActII
DN-TbIIR
DN-ActII & DN-TbIIR
pGL3
3TP-Luc
pMyoG-Luc

Figure 2

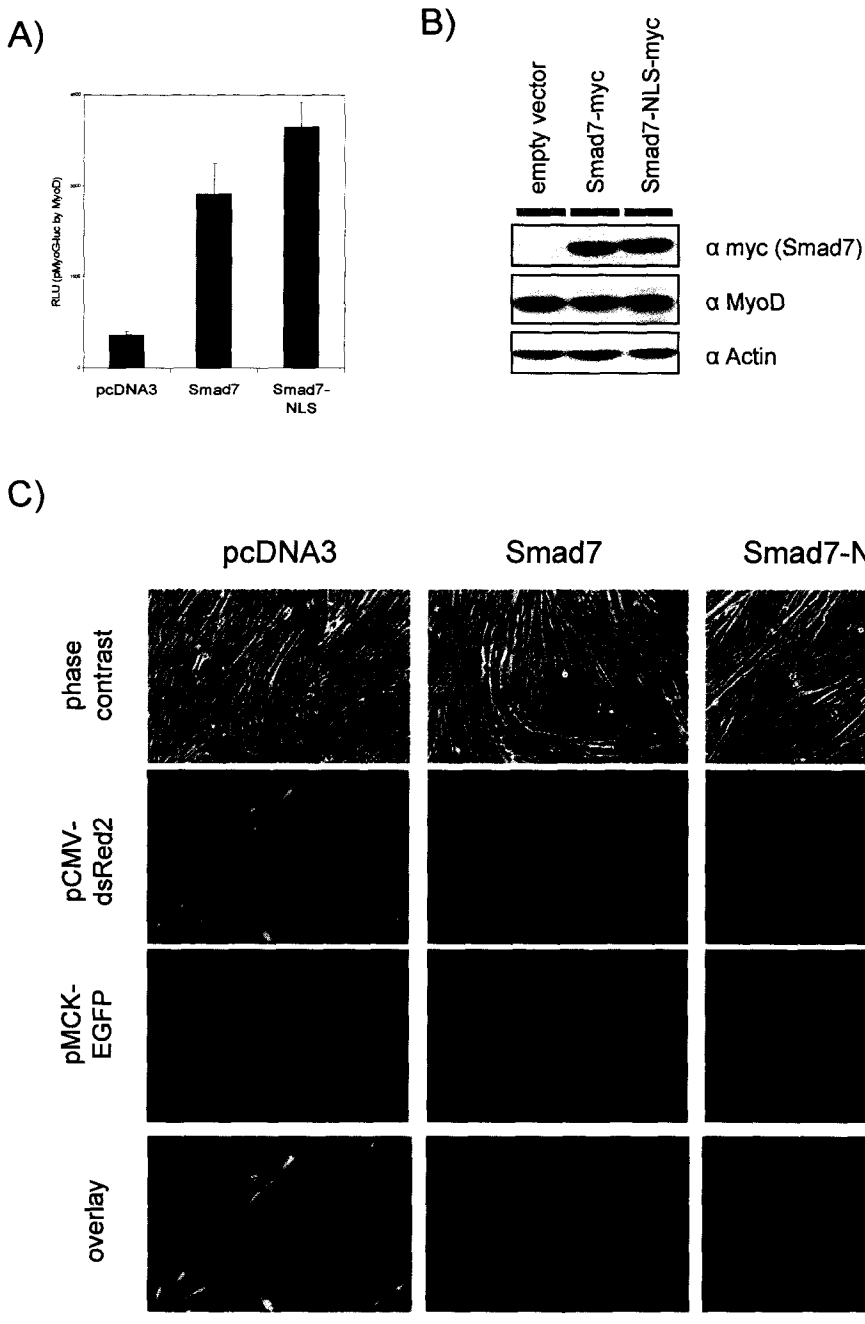

A)
RLU (pMyoGluc by MyoD)
pcDNA3
Smad7
Smad7-NLS
B)
empty vector
Smad7-myc
Smad7-NLS-myc
α myc (Smad7)
α MyoD
α Actin
C)
pcDNA3
Smad7
Smad7-NLS
phase contrast
pCMV-dsRed2
pMCK-EGFP
overlay

Figure 3

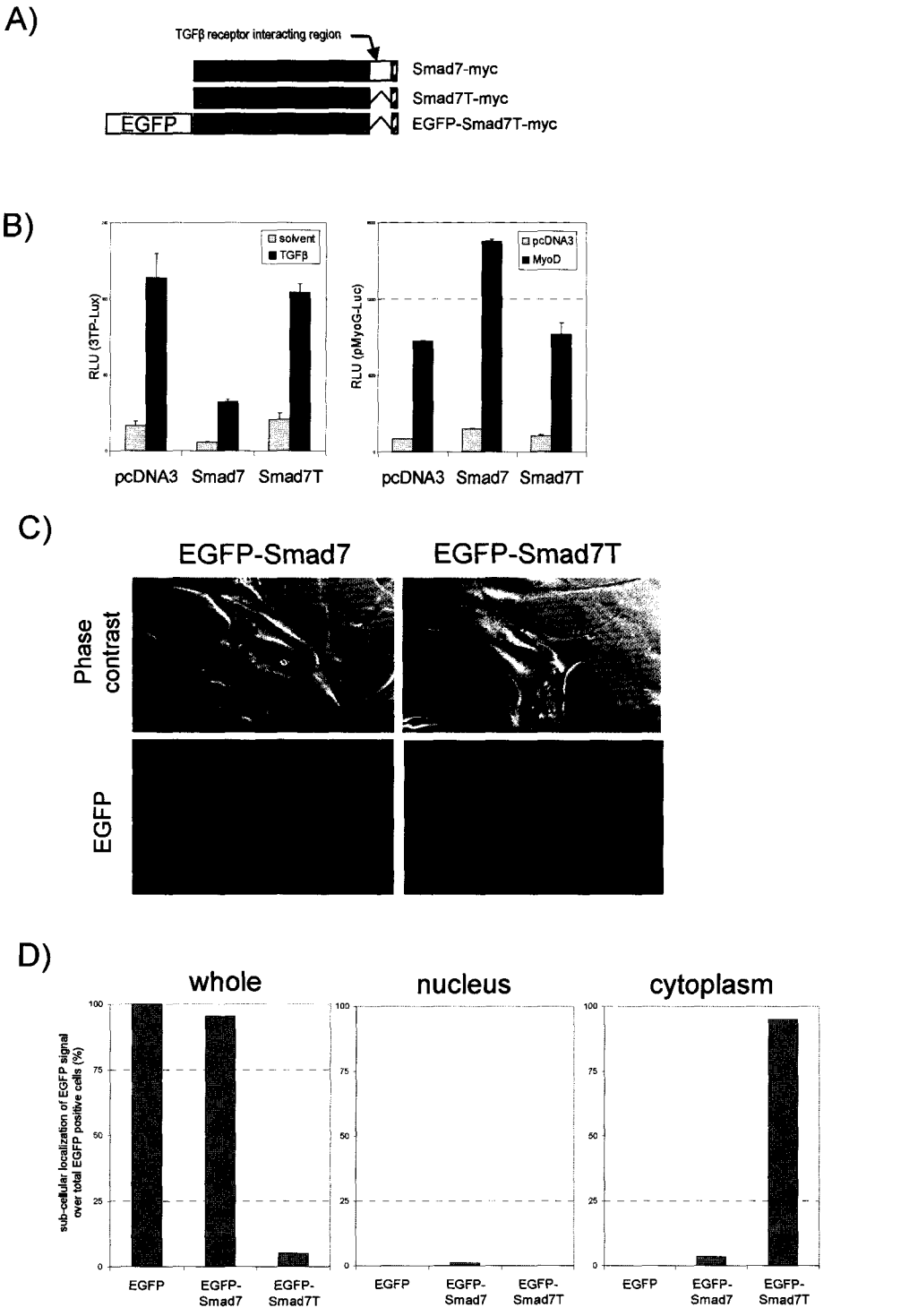

A)
TGFβ receptor interacting region
Smad7-myc
Smad7T-myc
EGFP
EGFP-Smad7T-myc
B)
solvent
TGFβ
RLU (3TP-Lux)
pcDNA3 Smad7 Smad7T
pcDNA3
MyoD
RLU (pMyoG-Luc)
pcDNA3 Smad7 Smad7T
C)
EGFP-Smad7
EGFP-Smad7T
Phase contrast
EGFP
D)
whole
nucleus
cytoplasm
sub-cellular localization of EGFP signal over total EGFP positive cells (%)
100
75
50
25
0
EGFP
EGFP-Smad7
EGFP-Smad7T

Figure 4

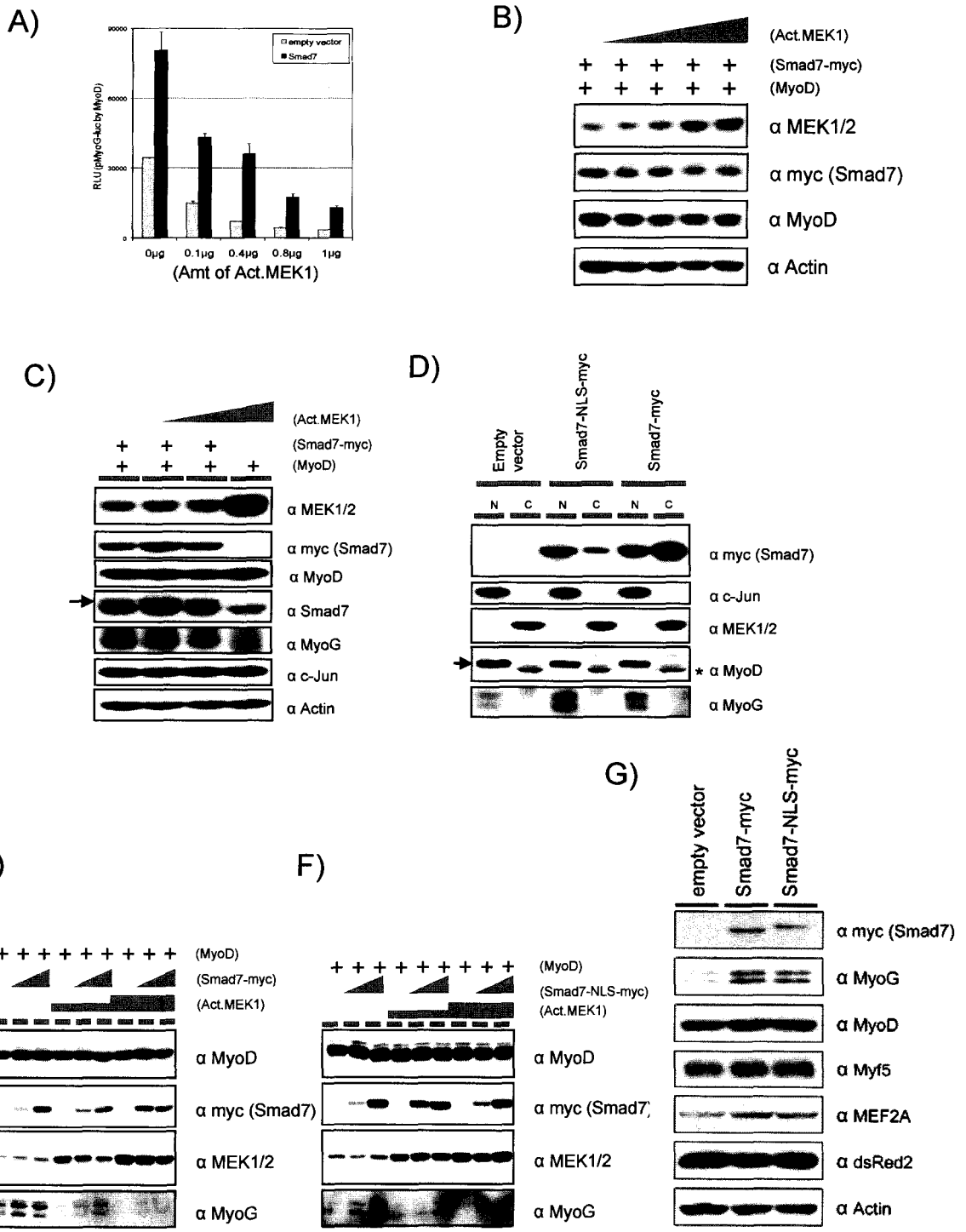

A)
empty vector
Smad7
RLU (pMyoG-luc by MyoD)
0μg
0.1μg
0.4μg
0.8μg
1μg
(Amt of Act.MEK1)
B)
(Act.MEK1)
(Smad7-myc)
(MyoD)
α MEK1/2
α myc (Smad7)
α MyoD
α Actin
C)
(Act.MEK1)
(Smad7-myc)
(MyoD)
α MEK1/2
α myc (Smad7)
α MyoD
α Smad7
α MyoG
α c-Jun
α Actin
D)
Empty vector
Smad7-NLS-myc
Smad7-myc
N C N C N C
α myc (Smad7)
α c-Jun
α MEK1/2
* α MyoD
α MyoG
E)
(MyoD)
(Smad7-myc)
(Act.MEK1)
α MyoD
α myc (Smad7)
α MEK1/2
α MyoG
F)
(MyoD)
(Smad7-NLS-myc)
(Act.MEK1)
α MyoD
α myc (Smad7)
α MEK1/2
α MyoG
G)
empty vector
Smad7-myc
Smad7-NLS-myc
α myc (Smad7)
α MyoG
α MyoD
α Myf5
α MEF2A
α dsRed2
α Actin

Figure 5

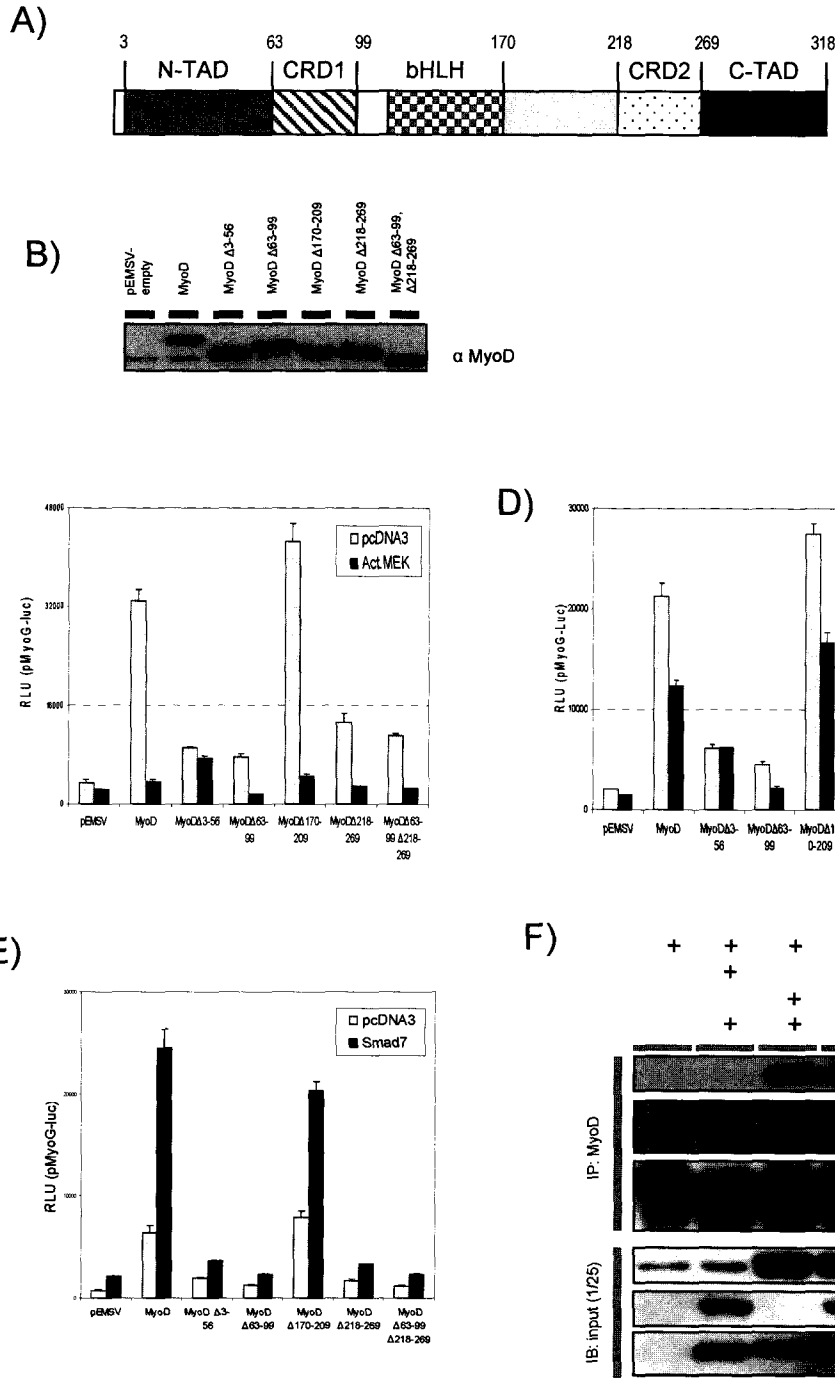
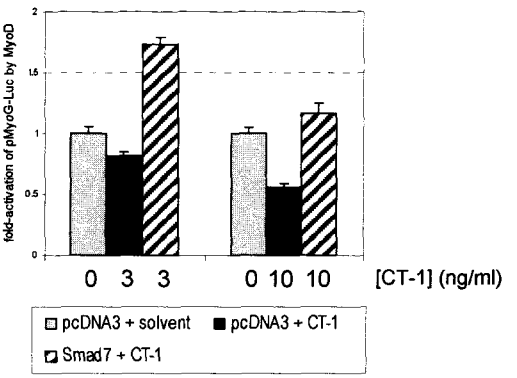

A)
3
63
99
170
218
269
318
N-TAD
CRD1
bHLH
CRD2
C-TAD
B)
pEMSV-empty
MyoD
MyoD Δ3-56
MyoD Δ63-99
MyoD Δ170-209
MyoD Δ218-269
MyoD Δ63-99, Δ218-269
α MyoD
C)
pcDNA3
Act.MEK
RLU (pMyoG-luc)
pEMSV
MyoD
MyoDΔ3-56
MyoDΔ63-99
MyoDΔ170-209
MyoDΔ218-269
MyoDΔ63-99 Δ218-269
D)
solvent
CT-1
RLU (pMyoG-Luc)
pEMSV
MyoD
MyoDΔ3-56
MyoDΔ63-99
MyoDΔ170-209
MyoDΔ218-269
MyoDΔ63-99 Δ218-269
E)
pcDNA3
Smad7
RLU (pMyoG-luc)
pEMSV
MyoD
MyoD Δ3-56
MyoD Δ63-99
MyoD Δ170-209
MyoD Δ218-269
MyoD Δ63-99 Δ218-269
F)
(empty vectors)
(Smad7-myc)
(Act.MEK)
(MyoD)
IP: MyoD
α MEK1/2
α myc (Smad7)
IgG
α MyoD
IB: input (1/25)
α MEK1/2
α myc (Smad7)
α MyoD

Figure 6

A)



B)

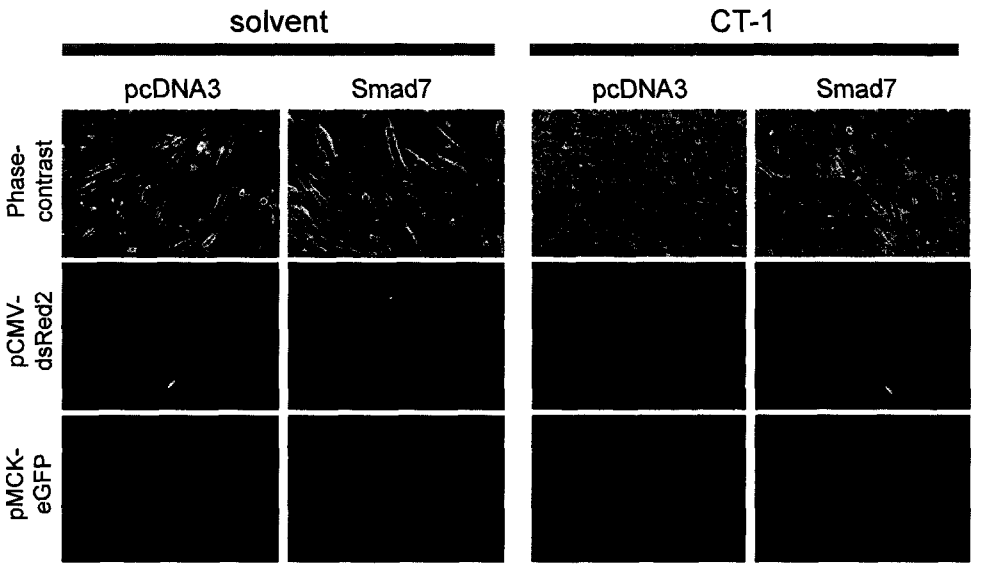


Figure 7

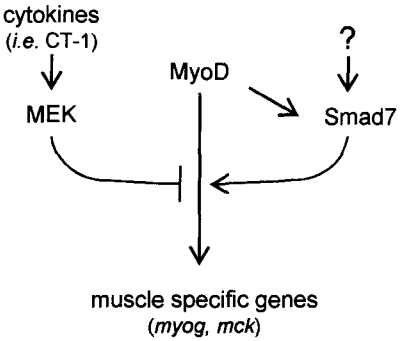


Figure 8

Chapter V; Maintenance of the undifferentiated state in skeletal myoblasts by TGFβ is Smad independent and requires MEK activation

Submitted to "Experimental Cell Research"

Experimental design and drafting manuscript

Tetsuaki Miyake and Dr. John C McDermott

Conducting experiments

Tetsuaki Miyake; (all figures except figure 1)

Dr. Arif Aziz (figure 1)

Title: Maintenance of the undifferentiated state in skeletal myoblasts by TGFβ is Smad independent and requires MEK activation

Tetsuaki Miyake, Arif Aziz, and John C. McDermott

Department of Biology, York University, 4700 Keele street, Toronto, Ontario, M3J 1P3 Canada.

correspondence to: John C.McDermott, Department of Biology, 327 Farquharson, LSB, York University, 4700 Keele St., Toronto, Ontario, Canada. M3J 1P3

Fax:(416)-736-5698, E-mail: jmcderm@yorku.ca

Running title: TGFβ inhibits myogenesis through activation of MEK signaling

Abstract (196)

Transforming Growth Factor β (TGFβ) is a pluripotent cytokine and regulates a variety of biological processes. It is well established that TGFβ potently inhibits skeletal muscle differentiation; however, the molecular mechanism of TGFβ's inhibitory effect is not well defined. We previously documented that inhibition of the TGFβ 'canonical' pathway by an inhibitory Smad, Smad7, does not reverse TGFβ's inhibitory effect suggesting that activation of receptor Smads (R-Smads) by TGFβ is not responsible for repression of myogenesis. In fact, pharmacological blockade of Smad3 activation by TGFβ did not reverse TGFβ's inhibitory effect. In considering other pathways, we observed that TGFβ potently activated MEK/ERK, and a pharmacological inhibitor of MEK

partially reversed TGFβ's inhibitory effect as indicated by a *myogenin* promoter-reporter gene, Myosin heavy chain accumulation, and myotube formation. Furthermore, a well established inhibitory molecule for myogenesis, the c-Jun protein, which is a down-stream target of MEK/ERK signaling, was phosphorylated and accumulated in the nucleus in response to TGFβ. In agreement with previous observations, activation of the *myog* promoter by MyoD was strongly inhibited by c-Jun. Taken together, these observations suggest that TGFβ activates a MEK/ERK/c-Jun pathway to repress myogenesis, thus, maintaining the pluripotent undifferentiated state in myoblasts.

Introduction (411)

TGFβ is the prototype of a large family of pluripotent cytokines with diverse effects on cellular proliferation, tumor growth, apoptosis, differentiation, anti-inflammation, and embryo development [1-5]. Competence to TGFβ cytokine signaling plays an important role in determining lineage acquisition in cells of mesenchymal origin, notably determining osteogenic or myogenic commitment [6]. The potency of TGFβ signaling in myogenic cells has been known for some time although dissection of the molecular pathway(s) is still fragmentary.

The general 'canonical' view that has been established for TGFβ signaling is that it binds to its cognate type II receptor which facilitates receptor complex formation and activation of the cytoplasmic serine/threonine kinase activity of the type I receptor leading to phosphorylation of Smad2/3 (receptor regulated Smads: R-Smads). Phosphorylation of R-Smads at the C'-terminal SXS motif results in

association with the common Smad, Smad4, and translocation into the nucleus to regulate target gene transcription through complex interactions with heterogeneous transcription complexes [7, 8]. While this pathway is pervasive in mediating TGFβ effects, a number of 'non-canonical' aspects of TGFβ signaling have also been reported [9, 10].

Previously, we observed that an inhibitory Smad (I-Smad), Smad7 potently counteracts Smad3 activation by TGFβ in a myogenic cell line. However, exogenous Smad7 was surprisingly not able to prevent the inhibition of muscle differentiation by TGFβ [11]. These observations indicate that TGFβ inhibits muscle differentiation through a Smad independent pathway. In further support for this idea, Myostatin, a member of the TGFβ family and a regulator of skeletal muscle differentiation, also activates Smad2/3 by phosphorylation of the SXS motif of the R-Smads in a manner analogous to TGFβ. However, exogenous expression of Smad7 reverses the inhibitory effect of Myostatin but not that of TGFβ [11]. Thus, several lines of evidence suggest that repression of myogenesis by TGFβ is mediated by a pathway distinct from the canonical 'R-Smad' pathway. Here, we document that TGFβ activates MEK-ERK signaling in response to TGFβ. MEK activation subsequently represses the transcriptional activity of MyoD [12-14]. Importantly, a MEK specific inhibitor, U0126 [15], reverses the inhibitory effect of TGFβ on myogenic differentiation, whereas pharmacological blockade of Smad3 signaling was without effect. These findings indicate that MEK, and not Smad3, activation is the primary mechanism underlying TGFβ's inhibitory action on myogenesis. These observations reveal the involvement of a TGFβ-MEK pathway

in maintaining myogenic precursor cells in the undifferentiated state and also place TGFβ at a strategic nexus to control the differentiation of pluripotent mesenchymal cells into different lineages.

Materials and Methods (859)

Plasmids Smad7 and Smad7T expression vectors were described previously [11]. An activated (ΔN3 S218D/S222E) human MEK1 expression construct was a gift from A.Natalie [16]. p3TP-Lux reporter construct was from J.Wrana (University of Toronto; Program in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute, Mount Sinai Hospital). (CAGA)X13-Luc reporter construct was generated by insertion of 13X (CAGA) sequence from the *pai-1* promoter [17] followed by a *c-fos* minimal promoter in the pGL3-basic (Promega) luciferase reporter vector. pCMV-β-galactosidase were described elsewhere [11]. The *myogenin* promoter region was excised from pMyoG-luc by *SacI /Bgl II* digestion. The resultant 1152bp fragment was inserted at the SacI */Bgl II* sites of pGL4-10 vector (Promega). The dsRed2-N1 expression vector was purchased from Clontech Laboratories. All constructs used in this study were verified by DNA-sequencing (York University Molecular Core Facility).

Antibodies The primary antibodies used in this study were obtained from Santa Cruz Biotechnology; MyoD (C-20), Actin (I-19); from Cell Signaling Technology; MEK1/2 (9122), Phospho-MEK1/2 (Ser217/221) (9121), STAT3 (9132), Phospho-STAT3 Y705 (9135), S727 (9136), Smad3 (9513), phospho-Smad3 (9514), Smad2

(3122), and Phospho-Smad2 (3101); from DakoCytomation; MyoD1 (clone:5.8A; M3512).

Cell Culture C2C12 myoblast and C3H10T1/2 were obtained from American Type Culture Collection and cultured in growth medium (GM) consisting of 10% Fetal bovine serum (FBS) (HyClone) in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 1% penicillin-streptomycin (Gibco) at 37°C and 5% CO_2. Myotube formation was induced by replacing GM with differentiation medium (DM) which consisted of 2% horse serum (Atlanta Biologicals) in DMEM supplemented with 1% penicillin-streptomycin. For TGFβ or CT-1 treatment, recombinant human TGFβ (R&D system; 240-B) or CT-1 (R&D system; 438-CT) was resuspended with solvent (4mM HCl, 0.1% bovine serum albumin (BSA)) and added into the media. For myotube formation assays, DM with TGFβ (1ng/ml) or CT-1 (10ng/ml) was replenished every 2 days. Inhibitors (PD98059 (Cell Signaling Technology; 9900), U0126 (Cell Signaling Technology; 9903), and SIS3 ((2E)-1-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-3-(1-methyl-2-phenyl-1H-pyrrolo[2,3-b]pyridin-3-yl)-propenone hydrochloride (Sigma-Aldrich, S0447) were resuspended with DMSO and added into the cell culture media for 30 minutes prior to adding TGFβ.

Microscopy Phase contrast photomicrographs were obtained using an epifluoresence microscope (Axiovert 35; Carl Zeiss MicroImaging), with appropriate phase and filter settings, and either 4X NA 0.10 or 10X NA 0.25

Achrostigmat objective lenses. Images were recorded with a digital camera (Canon, EOS D60).

Nuclear protein extraction Nuclear proteins were extracted from the cells by NE-PER® kit (Pierce) according to the manufacturer's protocol.

Western blotting analysis Total cellular protein extracts were prepared in NP-40 lysis buffer (0.1 % NP-40, 150 mM NaCl, 1mM EDTA, 50 mM Tris-HCl pH 8.0, 1mM sodium vanadate, 1mM PMSF, supplemented with a protease inhibitor cocktail (Sigma, P-8340)). Protein concentrations were determined by a standard Bradford assay (BioRad). Equivalent amounts of protein were resolved by SDS-PAGE gels, followed by electrophoretic transfer to an Immobilon-P membrane (Millipore) as directed by the manufacturer (Millipore). Blots were incubated with the indicated primary antibody in 5% milk in PBS or Tris buffered saline (TBS)-T (10mM Tris-HCl,pH8.0, 150mM NaCl, 0.1% Tween-20) or 5% Bovine serum albumin (BSA) in TBS-T according to the manufacturer's protocol at 4°C overnight with gentle agitation. After washing briefly, the blots were incubated with the appropriate HRP-conjugated secondary antibodies in 5% milk in PBS or TBS-T at room temperature according to the manufacturer's protocols (Santa Cruz Biotechnology, Cell Signaling Technology). After being washed three times with 1XPBS or 1XTBS (depending on the primary antibody) at room temperature, the blots were treated with the Enhanced chemiluminescence reagent (Amersham) to

detect immuno-reactive proteins. The blots were exposed to Biomax film (Kodak) for visual representation.

Immunochemistry C2C12 cells were washed with Phosphate buffered saline (PBS) (pH7.4) and fixed with 90% methanol at -20 °C for 10 min. After fixation, the cells were incubated in 5% milk in PBS for 30 min at 37 °C for blocking. Cells were incubated at room temperature with MF-20 (primary antibody) diluted in blocking buffer (5% milk PBS) for 1 hour. After incubation, the cells were washed three times with PBS and incubated for 60 min at room temperature with an Horseradish peroxidase (HRP)-conjugated α-mouse secondary antibody. The cells were again washed three times with PBS and incubated in developer (0.6 mg/ml DAB, 0.1 % H_2O_2 in PBS) to detect MyHC by immunocytochemistry. The nuclei were counter-stained with haematoxylin. Images were recorded with a microscope (Axiovert 35; Carl Zeiss MicroImaging) with either 4X NA 0.10 or 10X NA 0.25 Achrostigmat objective lenses with a digital camera (Canon, EOS D60).

Transcription reporter gene assays C2C12 myoblasts were transfected by a standard calcium phosphate-DNA precipitation method with the indicated reporter gene and expression constructs and pCMV-β-galactosidase to monitor transfection efficiency. After transfection, the cells were washed with PBS and maintained in GM and then treated as indicated. Total cellular protein was extracted with luciferase lysis buffer (20mM Tris-HCl pH7.4, 0.1% Triton X-100). Luciferase and β-galactosidase enzyme assays were performed according to the manufacturer's

protocol (Promega). Luciferase activity was quantified using a luminometer (Berthold Lumat, 9501) and standardized according to β-galactosidase activity. Relative Luciferase units normalized for β-galactosidase activity (Relative Luciferase Unit; RLU) were determined and plotted as an average of triplicate determinations and error bars represent standard deviations of the triplicate values.

Results (1119)

Inhibition of TGFβ mediated Smad3 phosphorylation does not reverse the inhibitory effect of TGFβ on muscle differentiation We previously found that although exogenous Smad7 strongly repressed Smad3 activation by TGFβ , the inhibition of muscle differentiation by TGFβ was not 'rescued' by exogenous expression of Smad7 [11]. These results suggested that TGFβ inhibits muscle differentiation in a Smad3 independent manner. To test this possibility, we inhibited Smad3 activation using a chemical inhibitor, SIS3 [18, 19]. In agreement with previous observations, SIS3 strongly repressed TGFβ induced phosphorylation of Smad3 (figure 1 A). In the absence of SIS3, TGFβ potently enhanced the activity of 3TP-Lux and (CAGA)X13-Luc reporter genes which are Smad3 dependent and TGFβ responsive reporter genes [17, 20]. The activation of these two reporter genes by TGFβ was strongly repressed by SIS3 indicating the efficacy of SIS3 inhibition of Smad3 activation (figure 1B). However, at the same concentration, at which SIS3 strongly inhibits phosphorylation and activation of Smad3, SIS3 failed to reverse TGFβ's inhibitory effect on muscle differentiation as assessed by myotube formation and myosin heavy chain (MyHC) accumulation (figure 1C). In

congruence with previous results, both SIS3 and Smad7 greatly reduced TGFβ induced Smad3 activity, but neither reversed TGFβ's inhibitory effect on muscle differentiation (figure 1C) [11]. Taken together, these observations strongly suggest that activation of Smad3 by TGFβ is insufficient for myogenic repression.

TGFβ stimulates MEK phosphorylation We hypothesized that if Smad3 activation is insufficient to inhibit muscle differentiation, TGFβ must activate a 'non-canonical' pathway to repress myogenesis. We noticed that TGFβ treated C2C12 cells reached high density and survived better in differentiation inducing medium (DM) (unpublished observation). In different systems, TGFβ has been observed to activate the MEK-ERK pathway [21, 22], and we therefore assessed the MEK-ERK pathway as a potential target for TGFβ signaling in muscle cells. Previously, we have documented that MEK activation is required for maintaining the undifferentiated state of myoblasts since a member of the IL-6 family, Cardiotrophin-1 (CT-1), inhibits myogenesis through MEK activation [23]. Therefore, we treated C2C12 cells with recombinant TGFβ as well as CT-1 as a positive control to assess phosphorylation levels of MEK.

Assessment of the MEK signaling pathway activation by immuno-blotting with antibodies recognizing the total and phosphorylated forms of MEK revealed that phosphorylated MEK was highly increased in C2C12 cells treated with TGFβ (2ng/ml) compared to that in solvent treated cells. TGFβ was more potent than CT-1 (10ng/ml) in terms of MEK activation (figure 2). Since we previously observed that CT-1 (10ng/ml) potently inhibits muscle differentiation [24] and the amount of

phosphorylated MEK due to TGFβ treatment was higher than that of CT-1, we reasoned that TGFβ mediated MEK activation in C2C12 cells could be sufficient to inhibit muscle differentiation. These results led us to postulate that TGFβ inhibits myogenesis by activation of the MEK signaling pathway.

Inhibition of MEK activation by a pharmacological inhibitor partially reverses the inhibitory effect of TGFβ on muscle differentiation We next tested the possibility that prevention of MEK activation by a MEK specific inhibitor might activate myotube formation and MyHC accumulation in the presence of TGFβ. As seen in figure 3A, C2C12 cells kept in DM for 72hrs without exogenous TGFβ formed large multinucleated myotubes, and these myotubes accumulated a molecular marker protein, MyHC (brown). In the presence of exogenously added TGFβ in DM, as previously observed by us and several other groups [25-27] , most of the cells maintained their mono-nucleated undifferentiated phenotype, and accumulation of MyHC was not observed (figure 3B). This undifferentiated phenotype with exogenous TGFβ administration was essentially reversed by treatment of the cells with the MEK inhibitor, U0126, in a dose-dependent manner (figure 3A). This indicates that MEK is a key down-stream target of TGFβ signaling, and the activation of MEK contributes considerably to the inhibition of muscle differentiation.

Reversal of TGFβ mediated myogenic repression by MEK inhibition is not due to inhibition of Smad signaling since MEK/ERK signaling may modulate R-Smad activity by phosphorylating the linker region of Smad2/3 [28-31]. In our experiments, MEK inhibition caused an increase in the activity of a TGFβ reporter

gene consistent with the idea that MEK/ERK inhibition de-represses the R-Smads [29, 31] (figure 3B). Thus, MEK inhibition does not rescue myogenesis by repressing TGFβ induced R-Smad activation. These data further support the idea that R-Smad activation is absolutely unnecessary for myogenic repression by TGFβ.

MEK specific inhibitors reverse the repression of MyoD transcriptional activation properties by TGFβ If MEK phosphorylation and subsequent nuclear accumulation are required for inhibition of muscle differentiation by TGFβ, we next tested whether MEK specific pharmacological inhibitors might reverse TGFβ mediated repression of MyoD transcriptional activation properties [13] quantified by the transcriptional activation of the *myog* gene which is a critical MyoD target gene in the hierarchical control of myogenesis [32-34]. To assess this, a *myog* promoter-luciferase reporter gene (pMyoG-luc) was used. C2C12 cells were transfected with this reporter gene construct and a MyoD expression vector. The transfected cells were treated with a MEK specific inhibitor, PD98059, or DMSO (diluent), and TGFβ protein or its solvent. As a positive control, CT-1 treatment was included in this analysis. TGFβ as well as CT-1 reduced MyoD driven *myog* promoter activation (figure 3A). In the presence of PD98059 (10μM), neither TGFβ nor CT-1 was able to inhibit transcriptional activity of MyoD efficiently suggesting the requirement for MEK signaling for myogenic repression by CT-1 and TGFβ (figure 3C). Therefore, these results indicate that TGFβ mediated MEK activation is required for the inhibition of MyoD's transcriptional properties, and inhibition of a primary myogenic target gene, the *myog* gene.

TGFβ signalling modulates MyoD co-activator and co-repressor proteins.

Previously, we reported that Smad7 can physically and functionally co-operate with MyoD in promoting myogenesis [11]. In this study, we document that Smad7 protein level is reduced by TGFβ treatment (figure 4A). Since MyoD can bind to and activate the Smad7 promoter [11], this effect is likely mediated by interference with MyoD transcriptional properties and repression of this positive feed-forward loop by TGFβ. Secondly we also documented that TGFβ signaling enhances the nuclear levels of phospho-c-Jun, a well established target of activated MEK signaling and a co-repressor of MyoD function [12, 14]. Our data analyzing the *myog* promoter confirms this repression of MyoD by c-Jun and also indicates that exogenous Smad7 expression cannot override this repression (figure 4A and B) consistent with Smad7's inability to inhibit TGFβ mediated myogenic repression [11].

Therefore, the primary inhibition of MyoD properties by TGFβ induced MEK activation is re-inforced by the subsequent down-stream inactivation of MyoD co-activators such as Smad7, and induction of MyoD co-repressors such as c-Jun. We suggest that these molecular events constitute a mutually re-inforcing network to lock the cells in an undifferentiated state.

Discussion (561)

It is widely assumed that TGFβ inhibits skeletal muscle differentiation via activation of the 'canonical' TGFβ/Smad3 pathway. However, we have made several key observations to dispute this idea. First, Smad7, an inhibitory Smad,

reversed the inhibitory effect of Myostatin but not TGFβ on myogenesis even though Smad7 potently inhibits Smad3 activation induced by both Myostatin and TGFβ [11]. Second, although a Smad3 specific inhibitor, SIS3, potently inhibited TGFβ induced Smad3 phosphorylation and subsequent activation of TGFβ/Smad3 dependent gene expression, it did not reverse the inhibitory effect of TGFβ on myogenesis. Third, a MEK inhibitor reverses TGFβ's inhibitory effect on myogenesis suggesting a 'non-canonical' pathway for myogenic repression. Fourth, a nuclear Smad7, which is incapable of inhibiting the TGFβ/Smad3 'canonical' pathway, is sufficient to enhance myogenesis (in press). These observations clearly indicate that TGFβ inhibits muscle differentiation independent of activation of R-Smads.

Is MEK activation a commonly used effecter of TGFβ family cytokines?

TGFβ is known to activate the MEK/ERK pathway [21, 35], and we recently found that this MAPK pathway plays an important role for maintenance of the undifferentiated state of myogenic precursor cells by CT-1 [23] and now, in the current study, by TGFβ. Interestingly, a recent study documented that BMP4, which is a known inhibitor of myogenic cell specification during embryo development [36] and a member of the TGFβ superfamily, induces neuronal differentiation in a Ras/ERK dependent manner [37]. Thus, these observations indicate the possibility that TGFβ cytokines commonly invoke MEK activation to restrict or promote lineage acquisition depending on the context. Further analysis of this idea is therefore warranted.

A detailed analysis of Smad independent TGFβ target genes may be enlightening in documenting these non-canonical effects of TGFβ signaling. Consistent with our observations, it was reported that *c-jun, junB*, and *smad7* genes are regulated by TGFβ in a Smad4 independent manner [38]. These data predict that regulation of *c-jun, junB*, and *smad7* gene expression by TGFβ would not be affected by inhibition of R-Smad activity although this remains to be tested. Furthermore, exogenous Smad7 expression could not reverse TGFβ's inhibitory effect on Insulin-like growth factor binging protein-5 (IGFBP5) synthesis in C2C12 MBs [39].

The identification of MEK as a potent effecter of TGFβ signaling in myogenic cells will allow this pathway to be manipulated pharmacologically in a variety of contexts. In particular, programming of multipotent mesenchymal cells or stem cells will greatly benefit from the characterization of signaling pathways that can repress or promote specific differentiation pathways in order to allow systematic programming of cells. In this regard, we envision that TGFβ, as a repressor of myogenesis, can be manipulated in favor of other lineages such as osteogenesis. Also, based on our studies repression of TGFβ induced MEK signaling would likely be a pre-requisite to promoting myogenic specification of precursor cells. Targeting the TGFβ/MEK cascade with cell permeable small molecule inhibitors has already proven to be a highly efficacious way to achieve some of these goals.

In summary, we have characterized the mechanism of TGFβ's inhibitory effect on myogenesis at the molecular level. TGFβ mediated repression of myogenesis is dependent on MEK activation and repression of MyoD activity. TGFβ effects on myogenesis are surprisingly but unequivocally independent of R-Smad activation.

Molecular dissection of TGFβ effects on myogenesis will allow further insights into the role played by this complex cytokine during development and post natal physiology in skeletal muscle.

Acknowledgements

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Figure legends (660)

Figure 1. **Inhibition of TGFβ mediated Smad3 phosphorylation does not reverse the inhibitory effect of TGFβ on muscle differentiation** A) C2C12 cells were seeded onto cell culture plates at equal density and maintained in TGFβ (1ng/ml) with or without indicated concentrations of SIS3. Total protein samples were extracted from the cells and equal amounts of total protein (20μg) were subjected to Western blotting analysis. The levels of indicated proteins were assessed by a standard immuno-blotting technique with a specific primary antibody. Actin indicates equal amounts of protein loading into each lane. B) C2C12 cells were transfected with either 3TP-lux (left panel) or (CAGA)X13-luciferase reporter gene construct (right panel), and to monitor transfection efficiency, pCMV-β-gal construct was included in each condition. The transfected cells were maintained for 16 hrs in TGFβ (1ng/ml) with or without indicated concentrations of SIS3. Total protein samples were harvested with a luciferase lysis buffer. Luciferase activity in each condition was measured independently and normalized according to β-Galactosidase activity. (n=3, +/- Stdv). C) C2C12 cells were seeded onto cell culture plates at equal density and maintained in TGFβ (1ng/ml) with or without indicated concentrations of SIS3 for 48hrs. The cells were fixed and stained for muscle myosin heavy chain (MyHC) detection by immunochemistry. The photomicrographs are representative fields in each condition.

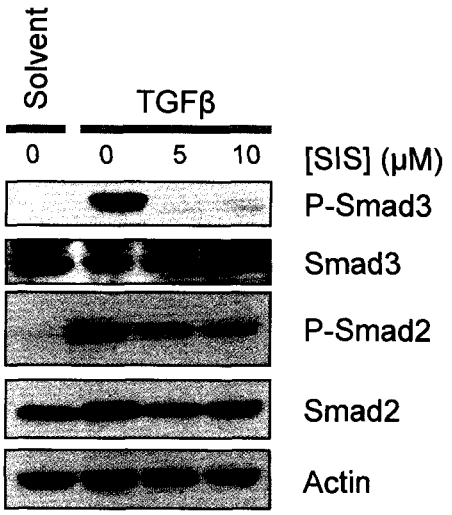
Figure 2. **TGFβ stimulates MEK phosphorylation** C2C12 cells were seeded onto cell culture plates at equal density and maintained in TGFβ (1ng/ml), CT-1 (10ng/ml), or solvent. Total protein samples were extracted from the cells and equal amounts of total protein (20μg) were subjected to Western blotting analysis. The levels of indicated proteins were assessed by a standard immuno-blotting technique with a specific primary antibody. Actin indicates equal amounts of protein loading into each lane.

Figure 3. **Inhibition of MEK activation by a pharmacological inhibitor partially reverses inhibitory effect of TGFβ on muscle differentiation** A) C2C12 cells were seeded onto cell culture plates at equal density and maintained in TGFβ (1ng/ml) or solvent with or without indicated concentrations of U0126 for 72 (left panel) and 96hrs (right panel). The cells were fixed and stained for muscle myosin heavy chain (MyHC) detection by immunochemistry. The photomicrographs are representative fields in each condition. B) C2C12 cells were transfected with (CAGA)X13-luciferase reporter gene construct, and to monitor transfection efficiency, pCMV-β-gal construct was included in each condition. The transfected cells were maintained for 16 hrs in TGFβ (1ng/ml) or solvent with or without indicated concentrations of U0126 (left panel) or PD98059 (right panel). Total protein samples were harvested with a luciferase lysis buffer. Luciferase activity in each

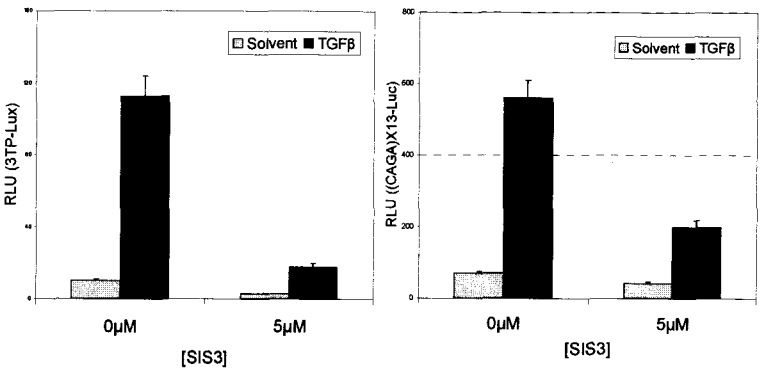
condition was measured independently and normalized according to β-Galactosidase activity. (n=3, +/- Stdv). C) C2C12 cells were transfected with myogenin promoter-luciferase reporter gene construct (pMyoG-Luc) with MyoD expression vector, and to monitor transfection efficiency, pCMV-β-gal construct was included in each condition. The transfected cells were maintained for 16 hrs in TGFβ (1ng/ml), CT-1 (10ng/ml), or solvent with or without indicated concentrations of PD98059. Total protein samples were harvested with a luciferase lysis buffer. Luciferase activity in each condition was measured independently and normalized according to β-Galactosidase activity. (n=3, +/- Stdv).

Figure 4. **TGFβ signaling modulates MyoD co-activator and co-repressor proteins** A) C2C12 cells were seeded onto cell culture plates at equal density and maintained in TGFβ (1ng/ml) or solvent in DM for 16hrs. Nuclear protein was extracted by using NE-PER®. The amount of indicated nuclear protein was visualized with standard Western blotting technique. Equal protein loading was monitored by c-Jun immunoblot. B) C2C12 cells were transfected with myogenin promoter-luciferase reporter gene construct (pMyoG-Luc) with MyoD expression vector, and indicated amount of c-Jun expression vector and combinations with Smad7 expression vector (0, 0.5, and 1μg). In addition, to monitor transfection efficiency, pCMV-β-gal construct was included in each condition. The transfected cells were maintained for 16 hrs in DM. Total protein samples were harvested with a luciferase lysis buffer. Luciferase activity in each condition was measured independently and normalized according to β-Galactosidase activity. (n=3, +/- Stdv).

A)


Solvent
TGFβ
0
0
5
10
[SIS] (μM)
P-Smad3
Smad3
P-Smad2
Smad2
Actin

B)


Solvent
TGFβ
RLU (3TP-Lux)
0μM
5μM
[SIS3]
RLU ((CAGA)X13-Luc)
0μM
5μM
[SIS3]

C)

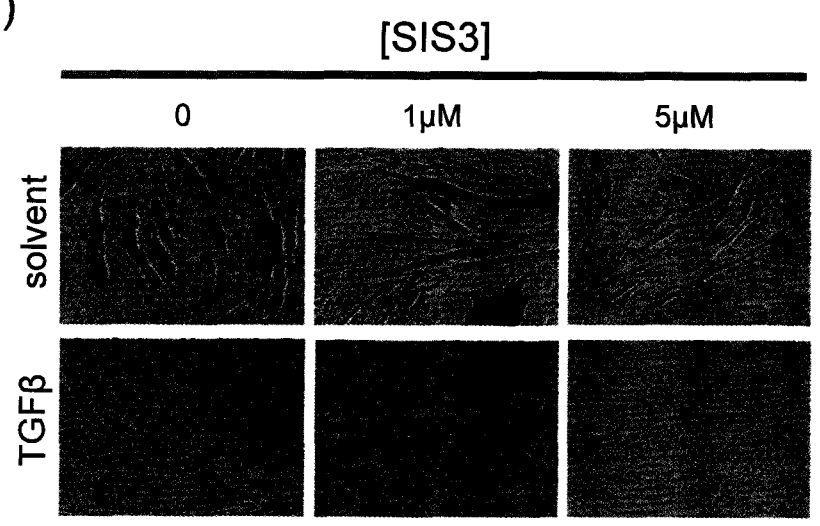

[SIS3]
0
1μM
5μM
solvent
TGFβ

figure 1

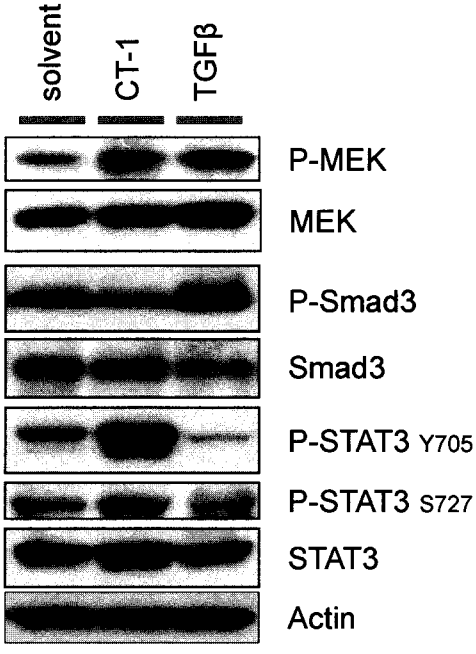
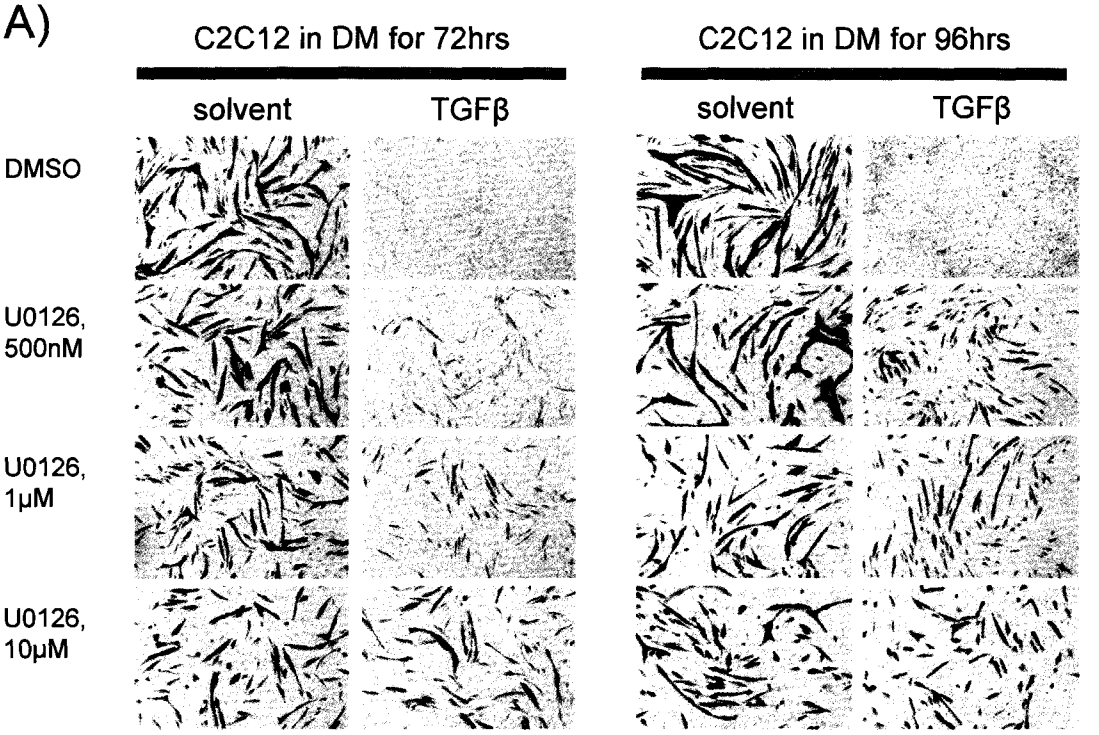
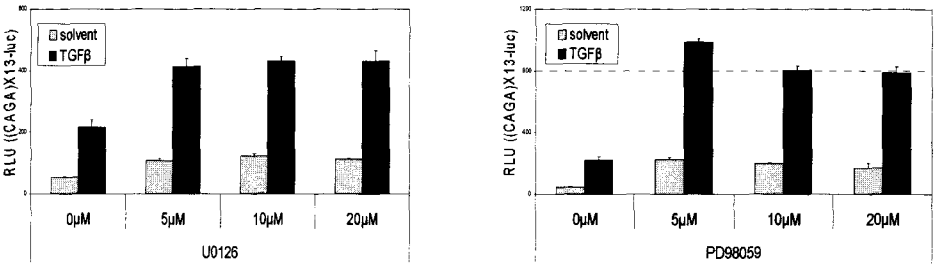

solvent
CT-1
TGFβ
P-MEK
MEK
P-Smad3
Smad3
P-STAT3 Y705
P-STAT3 S727
STAT3
Actin

figure 2

A)


C2C12 in DM for 72hrs
C2C12 in DM for 96hrs
solvent
TGFβ
solvent
TGFβ
DMSO
U0126, 500nM
U0126, 1μM
U0126, 10μM

B)


solvent
TGFβ
RLU ((CAGA)X13-luc)
0μM
5μM
10μM
20μM
U0126
PD98059

C)

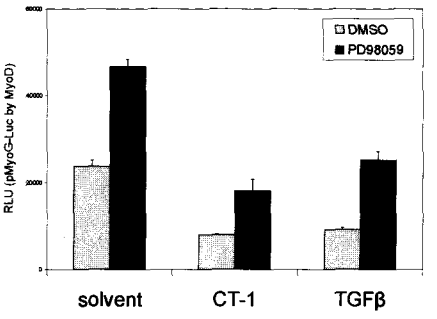
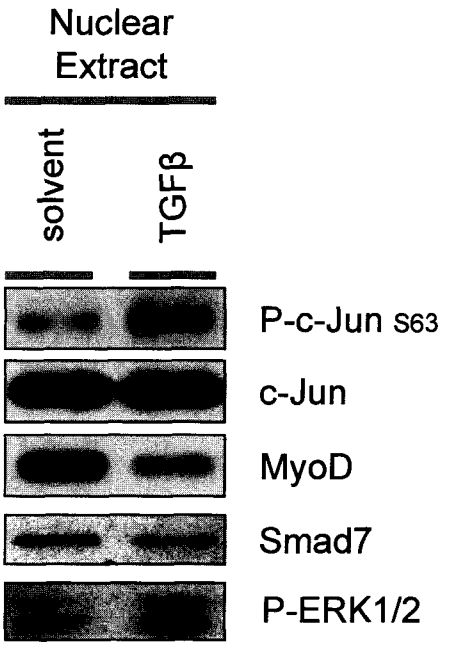

DMSO
PD98059
RLU (pMyoG-Luc by MyoD)
solvent
CT-1
TGFβ

figure 3

A)


Nuclear Extract
solvent
TGFβ
P-c-Jun S63
c-Jun
MyoD
Smad7
P-ERK1/2

B)

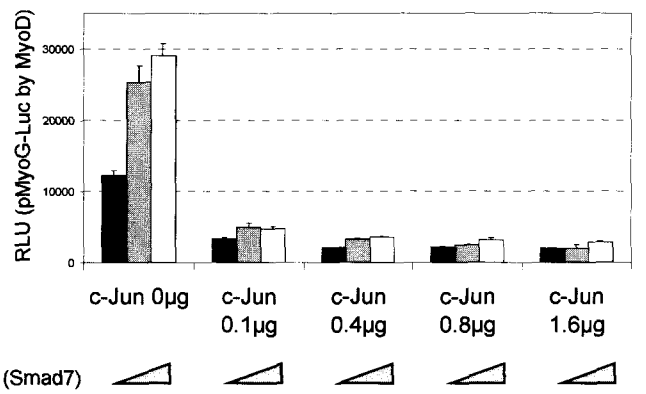

RLU (pMyoG-Luc by MyoD)
30000
20000
10000
0
c-Jun 0μg
c-Jun 0.1μg
c-Jun 0.4μg
c-Jun 0.8μg
c-Jun 1.6μg
(Smad7)

figure 4

Chapter VI; Conclusion

Conclusion

In this work I have studied the signal transduction pathways initiated by two physiologically important cytokines for skeletal muscle differentiation. In these studies I documented that as CT-1 and TGFβ, both repress the gene expression program leading to skeletal muscle differentiation. The inhibitory effect of both cytokines on muscle differentiation is mediated by convergence of the signalling on the activation of MEK1, a MAP kinase kinase. Activated MEK1 physically interacts with MyoD and inhibits MyoD's trans-activation properties. As a result, an essential process for myogenic differentiation, such as induction of the *myog* gene by MyoD, was found to be impaired in the presence of CT-1 or TGFβ. Therefore, we propose that these cytokines maintain the undifferentiated state of myoblasts and may serve an *in vivo* role to maintain myogenic precursor cells from premature differentiation.

Interestingly, Smad7, which was originally characterized as an inhibitory regulator of TGFβ/Smad3 signalling pathway, is essential for myogenic differentiation because in the nucleus Smad7 interacts with MyoD and antagonizes MEK1's inhibitory effect on MyoD's trans-activation properties, and Smad7, therefore, potentiates muscle differentiation. In summary, since not only CT-1 and TGFβ but also other cytokines and secreted proteins are known to activate MEK signalling and inhibit myogenesis, I propose that MEK1 activation is a nexus of myogenic regulation. Activated MEK1 by extracellular signalling and Smad7converge on MyoD and regulate the its trans-activation properties and therefore muscle lineage determination and differentiation (see Figure 32). Since loss of the muscle-mass is typical consequence of a variety

chronical disease conditions and also ageing, the elucidation of these pathways at the molecular level may lead to the development of pharmacological and/or physiological interventions that can alleviate or even prevent these pathophysiological conditions.

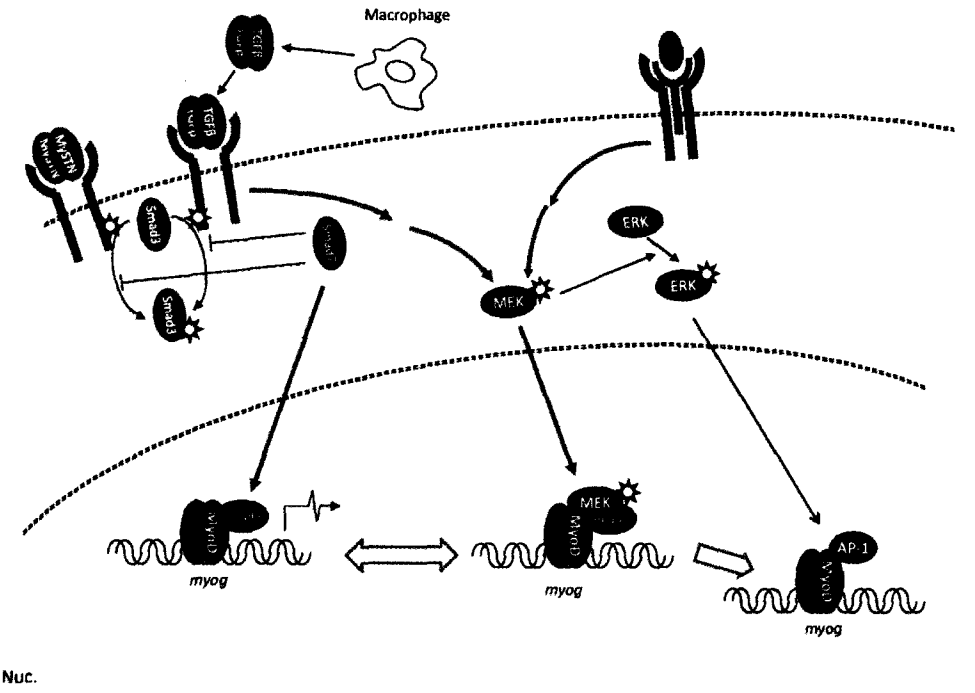


Figure 32 **This schematic depicts the major findings of these studies.** For details please see the text in the conclusion section above.

APPENDIX

APPENDIX

Molecular techniques;

Cell Culture

Reagents: 1x Dulbecco's PBS, Versene (0.2g of EDTA in 1L 1x PBS), 0.125% Trypsin-EDTA (Gibco) diluted in Versene, DMEM (supplemented with Penicillin-Streptomycin (Gibco) and L-glutamine (Gibco) added as required), Freezing medium ((Growth media (GM) supplemented with 10% DMSO); sterilize the freezing medium by passing through a 0.2um filter), FBS(heat inactivated at 56°C for 30 min), HS (heat inactivated at 56°C for 30 min).

Cell passaging

1. Remove media.
2. Rinse the cell monolayer with 4 ml of Versene.
3. Add 2.0ml of 0.125% Trypsin-EDTA solution to 100mm dish.
4. Remove the Trypsin-EDTA solution.
5. Add 10 ml of GM.
6. Pipette the cells up and down with the GM.
7. Plate cells accordingly.

Inducing Muscle Cell Differentiation

1. At 60-80% confluence, wash cells with PBS and re-feed with 5% HS in DMEM (differentiation medium (DM)).
2. Incubate cells for desired time at 37°C with 5% CO_2

Transfection of Mammalian Cells with DNA

Reagents: 2x HEBS (2.8 M NaCl, 15mM Na_2HPO_4, 50mM HEPES)(adjust pH to 7.15, filter sterilize, store at -20 °C), 2.5 M $CaCl_2$ (filter sterilize, store at -20 °C).

Calcium-phosphate transfection

1. Plate cells day before transfection for 30-50% confluent.
2. Re-feed cell cultures with GM 2-3 h prior to addition of DNA.
3. Label sterile tubes and add 0.5 ml of 2x HEBS to each tube.
4. Prepare DNA-$CaCl_2$ solution as follows, add 25 µg DNA, bring up volume to 450 µl, mix, add 50 µl 2.5 M $CaCl_2$, mix.
5. Add DNA-$CaCl_2$ solution drop-wise to the HEBS.
6. Add DNA mix drop-wise to cell cultures.
7. 16 h after addition of DNA, wash cells with 1XPBS and re-feed with GM.

Luciferase Assay

Reagents: Luciferase assay Lysis buffer (20 mM Tris, pH 7.4, 0.1% Triton-X 100), Luciferase substrate (Promega).

1. Wash adherent cells with 1XPBS.
2. Add 300 µl of lysis buffer per well/dish (35mm).
3. Harvest cells and spin-down cell debris at 15000rmp for 10min.
4. Transfer cell lysate into new tubes.
5. Transfer 30 µl to Luciferase assay tube.

β-Galactosidase Assay

Reagents: ONPG (4 mg/ml in ddH_2O), Z buffer (60 mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$), 1 M Na_2CO_3

1. Prepare reaction mixture (per sample (500 µl Z buffer, 100 µl ONPG, 2.74 µl β-mercaptoethanol)).
2. Incubate tubes at 37 °C until a color change is apparent (yellow).
3. Add 400 µl of 1M Na_2CO_3 to each tube to stop reaction.
4. Measure absorbance of samples at 420 nm.

Protein Extracts

Reagents: ice-cold 1XPBS, Lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM Sodium vanadate, 1 mM PMSF (add fresh), Protease inhibitor cocktail (add fresh, Sigma, P-8340), 2X SDS sample buffer (BioRad) (supplemented with β-mercaptoethanol)

1. Wash cells with ice-cold 1XPBS twice.
2. Scrape cells and transfer to a new tube.
3. Centrifuge at 1500XG for 2min.
4. Remove PBS, and re-suspend the pellet with five times (vol/vol) lysis buffer.
5. Vortex cells briefly every 10 min for 30 min on ice.
6. Centrifuge cell lysate at 10 000XG for 15 min, and transfer supernatant to new tube.
7. Determine protein concentration by Bradford assay.

Nuclear and Cytoplasmic Protein Extracts (NE-PER kit, Pierce)

1. Scrape cells and pellet by centrifugation at 1 500XG for 5 min at 4 °C.
2. Remove supernatant and add 200 µl of ice-cold CER I to the cell pellet.
3. Vortex the tube for 15 sec and then incubate tube on ice for 10 min.
4. Add 11 µl of ice-cold CER II to the tube.
5. Vortex the tube for 5 sec on the highest setting and then incubate tube on ice for 1 min.
6. Vortex the tube for 5 more sec and then centrifuge at 13 000xg for 5 min at 4 °C.
7. Immediately transfer the supernatant (cytoplasmic extract) fraction to a clean pre-chilled tube. Place this tube on ice until use or storage.
8. Re-suspend the insoluble pellet fraction from step 7 in 100 µl of ice-cold NER.
9. Vortex on the highest setting for 15 sec every 10 min for 40 min.
10. Centrifuge the tube at 13 000XG for 10 min at 4 °C and then transfer supernatant to new tube.
11. Determine protein concentration by Bradford assay and analyze samples by Western analysis.

SDS-PAGE

Reagents: 1.5M Tris pH 8.8, 30% acrylamide mix, 10% SDS, 10% APS, TEMED, Laemmli buffer.

1. Prepare resolving gel and then top with stacking gel.
2. Fill bottom and centre well of mini-gel apparatus with 1X Laemmli buffer.
3. Load samples on a gel.
4. Run a gel at 100-150 V.

Western blotting

1. Transfer protein from a gel to Immobilon-P (Millipore) membrane by wet-transfer at 20 V for 16 hrs.
2. Block membrane with 5 % (w/v) skim milk powder in 1XPBS/TBS (blocking solution).
3. Incubate membrane with primary antibody in blocking solution for 1-16 hrs at 4 °C.
4. Wash membrane with 1XPBS/TBST (3 X 5 min each).
5. Incubate membrane with secondary antibody in blocking solution for 1-2 hrs at room temperature (RT).
6. Wash membrane with PBS/TBST (3 X 5 min each).
7. Apply chemiluminescence reagent, and expose blot to film.

Co-Immunoprecipitation

1. Prepare cell lysates as described in protein extracts section.
2. Dilute protein sample in lysis buffer.
3. To 1 ml of cell lysate (250-1000µg total protein) add 1-5 µg of primary antibody and incubate at 4 °C for 1 h with gently agitation.
4. Add 30-50 µl of Protein G-Agarose, and nutate 16 hrs at 4 °C.
5. Pellet immuno-complex by centrifugation at 1000XG for 30 sec.
6. Wash pellet with 1 ml of lysis buffer.
7. Repeat steps 5 and 6 twice more.
8. Re-suspend pellet in 40 µl of 2 X SDS sample buffer and boil for 3 min, and transfer supernatant to new tube.
9. Sample ready for immuno-blotting.

Immunochemistry

Reagents: Fixative (90% ice-cold methanol), Blocking reagent (5 % skim-milk in 1XPBS).

1. Wash cells and fix and permeabilize with 90 % methanol for 10 min at -20°C.
2. Block with 5 % skim-milk in 1XPBS at 37 °C for 30 min.
3. Incubate cells with primary antibody for 1 hr in 5 % skim-milk.
4. Incubate cells with Horseradish peroxidase (HRP)-conjugated secondary antibody, 1 hr in 5 % skim-milk 1XPBS.
5. Wash cells three times with 1XPBS and incubated in developer (0.6 mg/ml DAB, 0.1 % H_2O_2 in 1XPBS).
6. Counter-stain nuclei with haematoxylin.
7. Wash several times in ddH_20.
8. Mounting cells with mounting media and cover-slip.

RNA Isolation

1. Add 1 ml of Trizol to 100 – 35 mm dish, agitate for 5 min and then transfer solution to microfuge tube.
2. Add 200 µl chloroform to cell suspension, vortex for 15 sec, and leave at RT for 2-3 min.
3. Centrifuge samples at 12 000XG for 15 min at 4 °C.
4. Transfer the aqueous phase to a fresh tube.
5. Add 500µl of isopropanol to the aqueous phase and incubate at RT for 10 min.
6. Centrifuge samples at 12 000XG for 10 min at 4 °C.
7. Remove the supernatant and leave pellet.
8. Wash RNA pellet with 70% ethanol.
9. Centrifuge samples at 7500XG for 5 min at 4 °C.
10. Remove supernatant and air dry for 5-10 min.
11. Dissolve the pellet in 25-50 µl of DEPC-treated water.

Cloning technique

Vector construction

1. Research the gene you want to express (if you can do sub-cloning, you may skip this step)

a. Go to PubMed, type a name of gene you want to clone, and choose "Gene" from a drop-down menu
b. Choose an appropriate species according to your template.
c. Examine sequences (isoforms, size, and date registered (*latest one is often better))
d. Copy and paste DNA sequence according to your plan (typically ATG to STOP: ORF) to Word. (*record accession # for future reference)
e. Copy and paste the sequence to WedCutter2.0 to identify the restriction sites you can use for cloning.
f. Examine G/C content (if it is more than 70%, you may need special DNA polymerase for PCR amplification)

2. Choose a vector based on the following

a. promoter (constitutive (CMV, SV40), or regulated (MCK, MyoG,...))
b. antibiotic resistance gene (ex. Amp-R (if not Amp-R, you may need to prepare bacterial plates)
c. screening drug (G418, puro, ...)
d. sequencing feature (T4, SP6, CMV, BGH, if Lee do not have one, you need to provide it)
e. multiple cloning sites (directional insertion, double digestion, Methyl-sensitive, restriction enzyme...)

3. Design a set of primers for PCR

a. chose DNA polymerase (ex. *Phusion* for a normal template).
b. Set-up an annealing temperature.
c. a forward primer needs 1-3 Nts for following restriction digestion, a restriction site of your choice, Kozak sequence for optimal translation (if no tag), ATG (You may eliminate the ATG of the ORF if you want to add a tag or peptide (Gal4-DBD, GFP, GST...)).
d. A reverse primer needs reverse-complement of the 3'end of the ORF (STOP codon MUST be eliminated fro the primer to put tag or peptide at C,-terminus), a restriction site of your choice, and 1-3 Nts to facilitate the restriction enzyme binding to PCR products.

4. Digest Vector DNA accordingly, and Gel-purify the linearised vector DNA. (If you can do directional ligation (two different cohesive ends), you should not do CIP treatment. However, you need to do non-directional ligation; you MUST do CIP treatment with your digested vector DNA ONLY, NOT insert.)

5. Amplify insert DNA by PCR

   a. Prepare template DNA (by RT-PCR from mRNA, gDNA or a vector you have).
   b. Set-up PCR reaction according to the manufacture's protocol.

6. Run PCR products in a DNA agarose gel to examine the size of PCR products.

7. If your PCR products have expected size, cut out DNA bands and purified DNA from the gel.

8. Digest purified DNA with appropriate restriction enzyme(s) (typically @37C for 1hr).

9. Set-up ligation reactions.

   a. total amount of DNA should be about 100ng.
   b. stoichiometry ratio between insert and vector DNA should be more than 1.
   c. 20μl in total reaction volume (1μl T4ligase).
   d. Allow ligase to seal DNA backbone at RT for more than 15 min.
   e. put on ice and cool-down tubes for transformation for a few minutes.

10. Set-up transformation

11. Examine the number of colonies on the plates.

12. Set-up mini-prep for screening. (number of colonies you would pick-up should be based on the ratio between number of colonies on **insert+vector** and **no-insert** plates.)

13. Do mini-prep and check insert size.

14. You may send the DNA from a positive clone for sequencing.