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Menin expression modulates mesenchymal cell commitment to the myogenic and osteogenic lineages

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ABSTRACT

Menin plays an established role in the differentiation of mesenchymal cells to the osteogenic lineage. Conversely, whether Menin influences the commitment of mesenschymal cells to the myogenic lineage, despite expression in the developing somite was previously unclear. We observed that Menin is down-regulated in C2C12 and C3H10T1/2 mesenchymal cells when muscle differentiation is induced. Moreover, maintenance of Menin expression by constitutive ectopic expression inhibited muscle cell differentiation. Reduction of Menin expression by siRNA technology results in precocious muscle differentiation and concomitantly attenuates BMP-2 induced osteogenesis. Reduced Menin expression antagonizes BMP-2 and TGF-β1 mediated inhibition of myogenesis. Furthermore, Menin was found to directly interact with and potentiate the transactivation properties of Smad3 in response to TGF-β1. Finally in concert with these observations, tissue-specific inactivation of *Men1* in Pax3-expressing somite precursor cells leads to a patterning defect of rib formation and increased muscle mass in the intercostal region. These data invoke a pivotal role for Menin in the competence of mesenchymal cells to respond to TGF-β1 and BMP-2 signals. Thus, by modulating cytokine responsiveness Menin functions to alter the balance of multipotent mesenchymal cell commitment to the osteogenic or myogenic lineages.

Introduction

Mesenchymal stem cells exist in a multipotent state until they commit to various cell lineages such as osteogenic and myogenic cell types. During ontogeny, differentiation of mesenchymal cells is regulated by integrated signaling networks which orchestrate changes in gene expression profiles leading to commitment to specific cell lineages (Asakura et al., 2001; Brand-Saberi et al., 1996). Myogenesis, the genetic process underlying the formation of multi-nucleated skeletal muscle cells (myotubes, MT) is a proven paradigm for cellular differentiation. Induction of the program for myogenic gene expression is critically dependent on transcription factors of the muscle regulatory factor family (MRFs) which is comprised of, MyoD, Myf5, MRF4 and Myogenin. The MRFs are members of the basic helix–loop–helix (bHLH) family of transcription factors, which integrate with ubiquitous transcription factors at the control regions of muscle-specific genes to promote myogenesis (Buckingham, 1992; Emerson, 1993; Lassar et al., 1994; Olson and Klein, 1994). Several myogenic genomic loci that have been well characterised in terms of their transcriptional circuitry, such as the muscle creatine kinase (*MCK*) and myosin heavy chain (*MyHC*) genes, serve as useful models to study the activation of tissue-specific gene expression during muscle differentiation (Jaynes et al., 1986; Sacks et al., 2003).

Osteogenic differentiation parallels myogenesis as it requires regulatory control of osteoblast-specific transcription factors such as the runt-related transcription factor Runx2/Cbfa1 (Runx2) and Osterix (Osx). The integration of pro-osteogenic transcription factors such as Runx2 with ubiquitous transcription factors and signaling pathway effectors regulates expression of osteoblast-specific genes such as alkaline phosphatase (*ALP*) and osteocalcin (*OCN*) (Komori et al., 1997; Nakashima et al., 2002; Otto et al., 1997). Thus, osteogenic and myogenic commitment of mesenchymal cells provides a useful system to study the commitment of multipotent progenitor cells into different cell lineages.

The interplay between extracellular signals and transcriptional regulation is a crucial nexus of control for cell lineage determination. Members of the transforming growth factor (TGF-β) superfamily of cytokines, including bone morphogenic protein-2 (BMP-2) and TGF-β1, play important roles during osteogenesis and myogenesis (Katagiri et al., 1990, 1994, 1997; Liu et al., 2001; Massague et al., 1986; Yamaguchi et al., 1991). Thus, competence to respond to the milieu of local growth factors is a fundamental determinant of the eventual fate of multipotent cells.

Abbreviations: MRF, muscle regulatory factor; MCK, Muscle creatine kinase; MyHC, Myosin heavy chain; Runx2/Cbfa1, Runt-related transcription factor; ALP, Alkaline phosphatase; BMP-2, Bone morphogenic protein-2; E, embryonic day; MB, myoblast; MT, myotube; e, enriched; r, reserve; GM, growth media; DM, differentiation media; CHX, cycloheximide.

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Changes in gene expression in response to the superfamily of TGF-β ligands, is most commonly regulated by ligand interaction with a transmembrane serine/threonine kinase receptor complex, resulting in phosphorylation-dependent activation of receptor-regulated Smads (R-Smads). The BMP regulated R-Smads are Smad1, -5, and -8; TGF-β regulated R-Smads are Smad2 and -3. Activated R-Smads interact with the common Smad (C-Smad), Smad4, and this complex translocates to the nucleus to regulate gene expression (Massague and Wotton, 2000). In addition to regulation of gene expression by co-operation with Smad4, R-Smads are implicated in the regulatory control of other key transcription factors such as, MyoD in muscle, and Runx2 in bone, during the regulation of lineage-specific gene expression (Lee et al., 2000; Liu et al., 2001). Thus, molecular integrators of these signals may be pivotal in mesenchymal lineage determination.

Commitment of mesenchymal cells into the osteogenic lineage and concurrent inhibition of myoblast differentiation into skeletal muscle is a property of BMP-2 signaling. TGF-β1 also has potent effects on mesenchymal cell differentiation and inhibits myogenesis (Katagiri et al., 1994, 1997; Massague et al., 1986; Olson et al., 1986; Yamaguchi et al., 1991). However, the question of how TGF-β signaling is differentially interpreted in homogeneous mesenchymal cell populations that will eventually commit to different lineages is not clear. Phosphorylation of Smad1/5 by the BMP-2 signaling cascade and interaction with Runx2 activates expression of pro-osteogenic genes such as *OCN* and *ALP* (Lee et al., 2000). TGF-β1 also potentiates BMP-2-mediated conversion of mesenchymal cells into an osteogenic phenotype through down-regulation of MRF protein expression and inhibition of myogenesis (Lee et al., 2000). The TGF-β1-effector Smad3 represses MyoD activity, although additional TGF-β1 effects are also evident in muscle cells (Liu et al., 2001). Thus, co-ordination of osteogenic and myogenic cell fates can be regulated by TGF-β1 and BMP-2 signaling. Interestingly, interaction of R-Smads with various other proteins such as Menin, confer the ability to modulate TGF-β1 and BMP-2 signaling in other cell types (Hendy et al., 2005; Kaji et al., 2001; Sowa et al., 2003, 2004a,b).

The C2C12 and C3H10T1/2 cell lines have been used as models to study the commitment of mesenchymal cells to the myogenic and osteogenic lineages. In C2C12 and C3H10T1/2 cell lines, BMP-2 mediates inhibition of terminal differentiation of cells into skeletal muscle and promotes an osteogenic phenotype (Katagiri et al., 1994, 1997). Previous reports implicate Menin, the protein product of the *Men1* gene, in the regulation of osteoblast differentiation in C3H10T1/2 cells, based on its capacity to modulate BMP-2 signaling. This effect is mediated by the interaction of Menin with Smad 1/5 and the key osteoblast transcription factor, Runx2 (Hendy et al., 2005; Sowa et al., 2003, 2004a). In osteoblasts, interaction of Menin with BMP-2 regulated Smads (Smad 1/5) is lost as committed osteoblasts differentiate further and Menin then interacts with Smad3 to negatively regulate Runx2 activity by TGF-β1 signaling (Sowa et al., 2004a). Furthermore, a previous report documented a role for Menin in osteogenic differentiation of Pax3-derived lineages as determined by *Pax3*-Cre driven deletion of Menin leading to defects in palatogenesis and rib formation (Engleka et al., 2007). Inactivation of Menin also blocks TGF-β1 signaling in anterior pituitary and parathyroid cells, reducing their growth inhibition by TGF-β1 (Hendy et al., 2005; Kaji et al., 2001; Sowa et al., 2004b). Interestingly, TGF-β1 and BMP-2 potently repress muscle cell differentiation although the role of Menin in modulating myogenesis has not so far been reported.

Men1 was first studied as a tumor suppressor gene, responsible for an autosomal dominant cancer syndrome, Multiple Endocrine Neoplasia type 1 (MEN1) which is characterised by multiple tumors of the parathyroid, endocrine pancreas, anterior pituitary and foregut-derived neuroendocrine tissues (Agarwal et al., 2004; Poisson et al., 2003). Menin is evolutionary conserved in various multi-cellular organisms including mouse (Stewart et al., 1998), rat, zebrafish (Khodaei et al., 1999) and *Drosophila* (Guru et al., 2001) with no known homologue in the yeast, *Saccharomyces cerevisae* (Poisson et al., 2003). Although the initial characterisation of Menin in MEN1 disease suggests a role as a tumor suppressor several roles have subsequently been postulated including a central role in transcriptional regulation, cell cycle control, apoptosis, genomic stability and differentiation. Such diverse functions are facilitated by a variety of interactions with cellular proteins such as: JunD, Smad3, MLL, Pem, Nm23, NF-κB, RPA2, and nmMHCIIa (Karnik et al., 2005; MacConaill et al., 2006; Milne et al., 2005; Poisson et al., 2003).

In mice, *Men1* transcript is detected during early development and is found in both non-endocrine and endocrine tissues as early as embryonic day 7 (E7). At a later gestational stage (E17), *Men1* expression is more restricted, with expression mainly confined to the thymus, skeletal muscle, brain and spinal cord. In contrast, analysis of adult mouse tissue reveals an absence of *Men1* transcript expression in skeletal muscle although it is present in numerous other tissues (Stewart et al., 1998). Interestingly, homozygous *Men1* null-mice die *in utero* between E11.5 and E13.5, and display delayed development with defects in multiple organs, including the neural tube, heart, liver, and cranial and facial formation (Crabtree et al., 2001). These disparate effects do not seem to be attributable to a general cell-autonomous effect. Therefore, a critical function for Menin has been proposed during development, in addition to its role in MEN1 disease (Crabtree et al., 2001; Sowa et al., 2003; Stewart et al., 1998).

In this report we document that reduction of Menin in mesenchymal cells leads to a precocious enhancement of differentiation into skeletal muscle and conversely reduces osteogenic differentiation. Menin directly potentiates Smad3 function and suppression of Menin expression antagonizes TGF-β1 and BMP-2 mediated osteogenesis and promotes myogenesis. Thus, we propose that Menin serves as a molecular rheostat, functioning to balance the ratio of commitment of mesenchymal cells to the myogenic and osteogenic lineages.

Materials and methods

Antibodies and reagents

The primary antibodies used for this study include: monoclonal and polyclonal antibodies purchased from Santa Cruz; α-Menin (C-19), α-GFP (B-2), α-dsRed (C-20), α-Myf-5 (C-20), α-MyoD (C-20), α-c-Jun (H-79), α-Actin (I-19), and α-ERK 1 (C-16); polyclonal antibody purchased from Bethyl Laboratories; α-Menin (BL-342); monoclonal antibodies purchased from BD Biosciences; α-MEF2D (610775), and α-Smad 2/3 (610842); monoclonal antibody purchased from Abcam; α-GAPDH (ab8245); polyclonal antibodies purchased from Cell Signaling Technology; α-p-Smad2 (3101), and α-p-Smad3 (9514); monoclonal antibodies purchased from Developmental Studies Hybridoma Bank; α-myogenin (F5D), and α-MyHC (MF20); monoclonal antibody purchased from Sigma; α-Flag (M2); monoclonal antibody purchased from R&D Systems; α-TGF-β1 (MAB1835). Polyclonal antibody α-MEF2A was prepared as previously described (Cox et al., 2003). Normal mouse (sc-2025) and goat (sc-2028) IgGs were purchased from Santa Cruz. Recombinant human BMP-2 (355-BEC) and TGF-β1 (240-B) were purchased from R&D Systems. Puromycin, MG132, CHX, and SIS3 were purchased from Sigma for use in cell culture. All other reagents were obtained as indicated herein.

Cell culture

Mouse C2C12 myoblasts and C3H10T1/2 embryonic fibroblasts were purchased from American Type Culture Collection (CLR-1772, CCL-226) and maintained in growth medium (GM) consisting of 10% FBS (HyClone) in high-glucose DME (Gibco) supplemented with 1% penicillin–streptomycin (Gibco) in a humidified environment at 37 °C