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CONTENTS

Chapter 1

Introduction

Beef marbling is characterized by adipose tissue deposition within skeletal muscle in cattle and is one of the important meat quality traits that influences juiciness and flavor of beef, which contributes directly to the value of beef on especially Japanese market. It has been accepted that high-energy grain diets achieve higher marbling than pasture diets (Price and Berg, 1981). The lipid content of beef depends on breed, sex, diet, and length of fattening (Cianzio et al., 1985; Gills and Eskin, 1973; Price and Berg, 1981). Japanese black cattle (Wagyu) are well known for its ability to produce high marbling (Lunt et al., 1993, Zenbayashi, 1994). The average marbling score according to the Japanese Carcass Grading Standards of the M. longissimus thoracis (LT) is 6.0. This value corresponds to approximately 17% intramuscular fat in LT (Cameron et al., 1994).

Amount of adipose tissue in an animal depend primarily on the number and of size of the constituent adipocytes (Waters, 1909). The increase in adipocyte number is considered to result from the proliferation and differentiation of preadipocytes, adipocyte precursor cells. Although the proliferation and differentiation of preadipocytes were completed in the perirenal and subcutaneous adipose tissues by the first year of age in cattle (Garbutt et al., 1979; Hood and Allen, 1973), intramuscular adipose tissue evidently was still actively growing by proliferation and differentiation in steers at 14 months of age

(Hood and Allen, 1973). Cianzio et al. (1985) also reported that the development of beef marbling was closely associated with an increase in adipocyte number within muscle during 13 to 19 months of age.

Many researchers reported the positive relationship between body fat accumulation and beef marbling in some foreign breeds of beef cattle (Riley et al., 2003; Wertz et al., 2001). On the other hand, some researchers showed no positive relationship between back fat depth and beef marbling in Japanese black cattle (Mukai et al., 1995; Yang et al., 1985). These results suggest that the systemic fat metabolism does not necessarily reflect the intramuscular fat deposition in this breed, therefore, locally produced factors may contribute to the development of adipocytes within muscle in Japanese black cattle. As intramuscular preadipocytes are surrounded by mature skeletal muscle myofibers, it is predicted that paracrine factors secreted from muscle fibres may regulate the differentiation of preadipocyte via intercellular interactions. The possibility of a direct regulatory interaction between myofibers and adipocytes was suggested in an early study by Jarett et al. (1985), who observed that skeletal muscle in insulin-treated rats secreted a paracrine agent (or mediator) which stimulated pyruvate dehydrogenase and glycogen synthase activity in adipocytes.

Muscle fibers secrete various factors, such as fibroblast growth factor (FGF), transforming growth factor-ß (TGF-ß), tumor necrosis factor-α (TNF-α), insulin-like growth factors (IGFs), hepatocyte growth factor (HGF), and interleukin-6 (IL-6) (Chargé et al., 2004). These cytokines are known to regulate the proliferation and differentiation of preadipocytes, as well as of myoblasts

(Chargé et al., 2004). Although IGF-1 promotes both proliferation and differentiation of preadipocytes, other growth factors and cytokines are generally considered as inhibitors of adipocyte differentiation (Choy et al., 2000, Ohsumi et al., 1994, Torti et al., 1989).

In most cell culture models, TGF-β is a potent inhibitor of adipocyte differentiation (Choy et al., 2000; Petruschke et al., 1994; Vassaux et al., 1994). TGF-β is expressed in cultured preadipocytes and in adipose tissue in vivo, however, transgenic overexpression of TGF-β in adipose tissue inhibits preadipocyte differentiation (Clouthier et al., 1997).

Activin is a multifunctional growth and differentiation factor that belongs to the TGF-β superfamily. Activin was discovered for its ability to regulate follicle-stimulating hormone (FSH) production by pituitary cells (Ling et al., 1986; Vale et al., 1986). Activin is a dimeric protein synthesized as a homo- or heterodimer of the district β subunits ($β_A$ or $β_B$), which combine to form activin A ($β_A$-$β_A$), activin B ($β_B$-$β_B$), or activin AB ($β_A$-$β_B$). Activin exerts its biological effects by interacting with four types of transmembrane receptors (type IA, IB, IIA, IIB) with protein serine/threonin kinase activity (Attisano et al., 1996). Activin $β_A$ subunit (Meunier et al., 1998; Tuuri et al., 1994) and activin receptors (Feijen et al., 1994) are expressed in many tissues throughout the body, and activin A is reported to act on many cell types including myoblast (Link and Nishi, 1997; Shiozuka et al., 1997). However, its action on preadipocyte differentiation is not still investigated.

Myostatin (growth/differentiation factor-8 (GDF-8)) is also a member of TGF-β superfamily that is essential for proper regulation of skeletal muscle mass (Lee

and McPherron, 1999). Mice carrying a targeted deletion of the gene encoding myostatin have a dramatic and widespread increase in muscle mass, the result of both hyperplasia and hypertrophy of muscle fibers, suggesting that myostatin normally acts as a negative regulator of muscle growth (McPherron et al., 1997). Mutation of myostatin gene also results in increasing skeletal muscle in certain breeds of cattle (Belgian Blue and Piedmontese), which is known as double muscling (Kambadur et al., 1997). On the other hand, the reduction of fat accumulation is observed in knock-out mice (Lin et al., 2002; McPherron and Lee, 2002) and in the double-muscled cattle (Kobolak and Gocza, 2002). Myostatin is first expressed in somites, in the myotome layer that gives rises to skeletal muscle (McPherron et al., 1997), and is highly expressed in skeletal muscle at later developmental stages and in adults. The expression of myostatin has been also detected in both fetal and adult heart, and adipose tissue, but the expression of myostatin is substantially lower in adipose tissue than in skeletal muscle (McPherron et al., 1997; Sharma et al., 1999). Myostatin exerts its biological function by binding to the activin receptors (Lee and McPherron 2001; Massagué and Chen, 2000), and is reported to inhibit the differentiation of mouse 3T3-L1 preadipocyte (Kim et al., 2001). However, the action of myostatin on bovine preadipocyte differentiation is still unknown. Furthermore, it is not clarified how myostatin regulates postnatal fat accumulation.

Follistain is a monomeric glycoprotein first isolated from ovarian follicular fluid on the basis of its ability to suppress FSH secretion by pituitary cells (Robertson et al., 1987; Ueno et al., 1987) Follistatin captures activin A and neutralizes its activity (Schneyer et al., 2003). Recently, follistatin is also

reported to capture myostatin, which prevents myostatin binding to activin receptor (Lee and McPherron, 2001). The studies on gene deletion (Matzuk et al., 1995) and overexpression (Lee and McPherron, 2001) of follistatin demonstrated that follistatin neutralized the inhibitory effect of its sensitive ligands on muscle development, and myostatin is considered to be an obvious candidate for the sensitive ligand. Follistatin is highly expressed in ovary, however, it is also widely distributed in non-gonadal tissues including muscle and adipose tissue (Schneider et al., 2000), predicting that the follistatin attenuate activin A and myostatin action on adipogenesis through forming the inactive complex in muscular and adipose tissue.

The purpose of this study was to clarify the regulation of preadipocyte differentiation by paracrine factors secreted from muscle cells. Especially, the author targeted the effect of the TGF-β superfamily members, activin and myostatin, and investigated their effect on the differentiation of bovine preadipocytes in stromal vascular cells derived from adipose tissue. The author also examined the mechanism of the inhibitory action of activin A on the differentiation of mouse 3T3-L1 preadipocyte.

Review of Literature

Beef Marbling

Beef marbling is characterized by adipose tissue deposition within skeletal muscle in cattle and is one of the important meat quality traits that influences juiciness and flavor of beef, which contributes directly to the value of beef on especially Japanese market. It has been accepted that high-energy grain diets achieve higher marbling than pasture diets (Price and Berg, 1981). The lipid content of beef depends on strains, sex, diet, and length of fattening. Japanese black cattle (Wagyu) is well known for its ability to produce high marbling (Lunt et al., 1993, Zenbayashi, 1994). The average slaughter age of Japanese Black cattle is 29.6 months, and 422 kg, respectively, in Japan. The average marbling score according to the Japanese Carcass Grading Standards of the M. longissimus thoracis (LT) is 6.0. This value corresponds to approximately 17% intramuscular fat in LT (Cameron et al., 1994).

Amount of adipose tissue in an animal depend primarily on the number and of size of the constituent adipocytes (Waters, 1909). It has been established that an increase in adipocyte number spontaneously occurs in mature animals if they are given high-energy diets (Faust et al., 1978; Miller et al., 1984). The increase in adipocyte number is considered to result from the proliferation and differentiation of preadipocytes, adipocyte precursor cells. Although the proliferation and differentiation of preadipocytes were completed in the perirenal

and subcutaneous adipose tissues by the first year of age in cattle (Garbutt et al., 1979; Hood and Allen, 1973), intramuscular adipose tissue evidently was still actively growing by proliferation and differentiation in steers at 14 months of age (Hood and Allen, 1973). Cianzio et al. (1985) also reported that the development of beef marbling was closely associated with an increase in adipocyte number within muscle during 13 to 19 months of age.

Many researches reported the positive relationship between body fat accumulation and beef marbling in some foreign breeds of beef cattle (Riley et al., 2003; Wertz et al., 2001). On the other hand, some researchers showed no positive relationship between back fat depth and beef marbling in Japanese Black cattle (Mukai et al., 1995; Yang et al., 1985). These results suggest that the systemic fat metabolism does not necessarily reflect the intramuscular fat deposition in this breed. Therefore, locally produced factors may contribute to the development of adipocytes within muscle in Japanese black cattle.

Development of myocyte and adipocyte

During mammalian development, the embryonic mesoderm gives rise to several highly specialized cell types, including skeletal myocytes, adipocytes, and chondrocytes (Cornelius et al., 1994; Taylor and Jones, 1979; Watt, 1991). The development of distinct cell types from multipotent mesodermal precursors can be viewed as a two-step process (Sager and Kovac, 1982; Weintraub et al., 1991). In the first step, termed commitment or determination, the developmental potential of the cell becomes limited to one particular lineage, be it adipose, muscle, or cartilage. In the second step, terminal differentiation, the cell

develops along its determined lineage to become a functional cell.

The C3H 10T1/2 cell line was established in 1973 from 14- to 17-day-old C3H mouse embryos (Reznikoff et al., 1973). These cells display fibroblastic morphology in cell culture and are functionally similar to mesenchymal stem cells. Treatment of C3H 10T1/2 cells with 5'-azacytidine, an inhibitor of mammalian DNA methylation, leaded to generate 25% of myocytes, 7% of adipocytes and 1% of chondrocytes (Konieczny and Emerson, 1984; Taylor and Jones, 1979). This in vitro model for development of three different cell types revealed that determination of the muscle and adipocyte lineages is controlled at the transcription level by a small number of tissue-specific transcription factors (Konieczny and Emerson, 1984). In muscle, the basic helix-loop-helix (bHLH) proteins MyoD and Myf-5 play an important role in lineage determination, while the related factors myogenin and MRF4 function to execute the differentiation program (Buckingham et al., 1992; Lassar and Munsterberg, 1994). In adipocytes, differentiation is controlled by two major transcription factors, CCAAT enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor (PPAR) γ, however, little is known about the regulation of the commitment into preadipocytes. Recently, Tang et al. (2004) reported that bone morphogenic protein (BMP) 4 causes the mesenchymal cells to undergo lineage commitment into preadipocytes. Further study is needed to clarify the regulatory genes that trigger the commitment process.

Although preadipocytes first appear in embryonic life, major expansion of the white adipocyte population is delayed until shortly after birth (Cook and Kozac, 1982; Slavin, 1979). The primary role of adipocytes is to store triglycerol during

periods of nutritional caloric excess and to mobilize this reserve when caloric expenditure exceeds. This increase in adipose tissue mass after birth coincides with the need of the newborn to survive periods of fasting by mobilizing adipose triacylglycerol reserves.

While the commitment of the pluripotent stem cells to preadipocytes and the differentiation into mature adipocytes also occur in postnatal stage, the postnatal growth of skeletal muscle is somewhat different from the development during early embryogenesis. Satellite cells, the primary stem cells in adult skeletal muscle, reside beneath the basal lamina of adult skeletal muscle closely juxtaposed against the muscle fibers, and are responsible for postnatal muscle growth, hypertrophy, and regeneration (Hawke and Garry, 2001). The actual origin of satellite cells is not known, but it is thought that they originate from generic myogenic cells, specific myoblast lineage, or remnant embryonic or fetal myoblasts (Dodson et al., 1996). Satellite cells are normally mitotically quiescent but are activated and re-enter the cell cycle in response to the growth factor released by the stimulus such as exercise or injury (Asakura et al., 2001; Bailey et al., 2001; Hawke and Garry, 2001). Once into the cell cycle, satellite cells undergo multiple rounds of division forming a pool of myogenic precursor cells, which ultimately undergo terminal differentiation and fuse to growing myofibers to form multinucleated myotubes (Asakura et al., 2001; Bailey et al., 2001; Hawke and Garry, 2001). As in the case during early development, myogenic regulatory factors such as MyoD, Myf-5, myogenin, and MRF-4 are involved in the activation, proliferation, and differentiation of satellite cells (Asakura et al., 2001).

Myoblast and preadipocyte differentiation in vitro systems

Various myoblast and preadipocyte cell lines have been established and used to study the conversion of myoblast into myotubes or preadipocyte into adipocytes.

Murine C_2C_{12} myoblast, which was established from normal adult C3H mouse leg muscle, is a well characterized myogenic cell line able to recapitulate myogenesis in vitro (Yaffe and Saxel, 1977). Proliferating C_2C_{12} myoblast, when cultured in a medium containing low concentration of mitogens, withdraw irreversible from the cell cycle, express muscle specific genes and fuse into mature myotubes. Although MyoD and Myf-5 are considered to be involved in the determination of the myogenic lineage, thy also appear to play an important role in initiating myoblast differentiation. In C_2C_{12} cells, MyoD, Myf-5, and MRF4 are expressed during the proliferation, while myogenin is only expressed after induction of differentiation (te Pas et al., 2000). MRF4 is considered to be involved in inducing myoblast proliferation (Hannon et al., 1992), and MyoD and Myf-5 is appeared to regulate the myoblast proliferation rate (Grounds et al., 1992; Koishi et al., 1995). After induction of differentiation by the medium containing low concentration of mitogens, MyoD and Myf-5 induce the expression of myogenin which leads to the terminal differentiation (Rundnicki et al., 1993).

The most extensively characterized and widely studied cell models for preadipocyte differentiation are the 3T3-L1 and 3T3-F442A lines. These lines were originally selected from disaggregated mouse embryo cells for their ability to accumulate cytoplasmic triacylglyderol lipid droplets (Green and Kehinde, 1974; 1975; 1976). Considerable evidence has been amassed that indicates that these cell systems represent faithful models for preadipocyte differentiation.

Protocols have been developed that can induce 3T3 preadipocytes to rapidly and synchronously progress through the differentiation program at high frequency (≥90%). The agents most widely used (often in combination) to differentiate 3T3-L1 preadipocytes and other preadipocyte cell lines include dexamethasone (a synthetic glucocorticoid agonist), high level of insulin (which act through the insulin-like growth factor-1 (IGF-1) receptor, methyl-isobutylxanthine (MIX; a cAMP phosphodiesterase inhibitor), and fetal bovine serum (Student et al., 1980). During the growth phase, 3T3-L1 and 3T3-F442A preadipocytes are morphologically similar to the fibroblastic preadipocytes cells in the stroma of adipose tissue. When induced to differentiate, 3T3 preadipocytes undergo several rounds of mitotic clonal expansion (Tang and Lane, 1999). The necessity of this mitotic event for the following adipogenesis is controversial (Qiu et al., 2001, Tang et al., 2003), however, the cells lose their fibroblastic character thereafter, assume a rounded-up appearance, and acquire the morphological and biochemical characteristics of adipocytes. PPARγ and several members of the C/EBP family of transcription factors participate in a signaling cascade (Cornelius et al., 1994; Gregoire et al., 1998; MacDougald and Lane, 1995) that culminates in the transcriptional activation of genes that produce the adipocyte phenotype. C/EBPβ is expressed immediately (within 2-4 h) after induction of differentiation. At this point in the differentiation program, however, C/EBPβ is unable to bind DNA (Tang and Lane, 1999) and thus cannot function as a transcriptional activator. Only after a long lag period (10-12 h) does C/EBPβ acquire DNA-binding activity (Tang and Lane, 1999), as the cells synchronously reenter the cell cycle and begin mitotic clonal expansion. Coincident with the

acquisition of DNA-binding activity, C/EBPβ binds to centromeres through consensus C/EBPβ-binding sites in centromeric satellite DNA (Tang and Lane, 1999). Having acquired DNA-binding function, C/EBPβ transcriptionally activates both the C/EBPα and PPARγ genes through C/EBP regulatory elements in their proximal promoters (Gregoire et al., 1998, Lane et al., 1999). C/EBPα and PPARγ coordinately activate the transcription of adipocyte specific genes, such as adipocyte fatty acid binding protein (aP2, Tontonoz et al., 1994), lipoprotein lipase (LPL, Schoonjans et al., 1995), phosphoenolpyruvate carboxykinase (PEPCK, Tontonoz et al., 1995), acyl-CoA synthase (Schoonjans et al., 1995), and uncoupling proteins (UCP-1 and -2, Medvedev et al., 2001; Sears et al., 1996). Although preadipocytes in vitro do not differentiate into unilocular adipocytes, subcutaneous injection of the cells in athymic mice leads to production of normal fat pads that are histologically indistinguishable from nature white adipose tissue (Green and Kehinde, 1979; Vannier et al., 1985). This suggests that preadipocyte cell lines are useful models to study adipogenesis in vitro.

Bovine preadipocytes derived from white adipose tissues

Although preadipocyte cell lines are certainly useful and convenient to clarify the mechanism of adipocyte differentiation, they suffer a disadvantage because they are aneuploid and often possess characteristics that differ from those of tissue preadipocytes. Therefore, it is important to verify findings obtained with immortalized cell lines by using diploid preadipocytes in primary culture.

A clonal bovine intramuscular preadipocytes (BIP) line was established from

the intramuscular white adipose tissue of the *Musculus longissimus thoracis* of Japanese Black cattle (Aso et al., 1995). Exponentially growing BIP cells exhibit a fibroblastic appearance. Small lipid droplets appear 5-6 days after adipogenic stimulation and occupy a large fraction of the cell volume at 10 days and beyond. Various changes have been shown to occur during the adipogenesis of BIP cells; DNA synthesis is decreased at 3days after stimulation, and an increase in glucose and acetate uptake is observed at 5 days after stimulation. The metabolism of BIP cells, which are derived from a ruminant, are different to that of 3T3-L1 cells. For example, this cell line has been shown not to express GLUT-4, an insulin-responsive glucose transporter, during adipogenesis. In addition, acetate is its main precursor for fatty acid synthesis.

The stromal-vascular (SV) cells prepared from adipose tissue are also used to study adipocyte differentiation. The SV cells can be isolated from various species, various postnatal stages, and various adipose depots, and contain not only preadipocytes but also other types of cells, such as macrophages, monocytes, fibroblasts, smooth muscular cells and endothelial cells (Hausman et al., 1980; Ramsay et al., 1992). Therefore, the SV cells may reflect the context in vivo. In the SV cell culture, the capacity of differentiation clearly depends on the donor and significantly decreases with ageing (Bjorntorp et al., 1982; Gregoire et al., 1995; Kirkland et al., 1990; Soret et al., 1999; Tchkonia et al., 2002). Molecular and biochemical characteristics of preadipocytes in SV cells have been well studied in many species, especially human and rodents (Gregoire et al., 1990; Lacasa et al., 1997a; Lacasa et al., 1997b; Masuzaki et al., 1995). On the other hand, the expression patterns and apparent function of the transcription factors

critical for preadiopocyte differentiation remains to be elucidated in bovine preadipocytes.

Myostatin

Myostatin (growth/differentiation factor-8 (GDF-8)) is a TGF-β family member that is essential for proper regulation of skeletal muscle mass (Lee and McPherron, 1999). Myostatin is expressed almost exclusively in cells of the skeletal muscle lineage, from embryonic myotome to striated muscle in adults. Mice carrying a targeted deletion of the gene encoding myostatin (Mstn) have a dramatic and widespread increase in muscle mass, the result of both hyperplasia and hypertrophy of muscle fibers, suggesting that myostatin normally acts as a negative regulator of muscle growth (McPherron et al., 1997). Mutation of myostatin gene also results in increasing skeletal muscle in certain breeds of cattle (Belgian Blue and Piedmontese), which is known to double muscling (Kambadur et al., 1997). The skeletal muscle of double muscled Belgian Blue cattle is ~20% greater than in normal- muscled cattle (Shahin and Berg, 1985). Myostatin is synthesized as a preprotein activated by two proteplytic cleavages. Removal of the signal sequence is followed by cleavage at a tetrabasic processing site, resulting in a 26kDa NH2-terminal propeptide and a 12.5 kDa COOH-terminal peptide, a dimmer of which is the biologically active portion of the protein. The myostatin sequence has been highly conserved through evolution (McPherron and Lee, 1997). Remarkably, the human, rat, murine, porcine, turkey, and chicken myostatin sequences are identical in the biologically active C-terminal portion of the molecule following the proteolytic processing site.

The mature peptide is reported to bind to the activin type II receptors, which leads to the intracellular signal transduction (Lee and McPherron, 2001; Massagué and Chen, 2000; Rebbapragada et al., 2003).

Activin

Activin is a multifunctional growth and differentiation factor that belongs to the transforming growth factor-β (TGF-β) superfamily. Activin is a dimeric protein hormone synthesized as a homo- or heterodimer of the district β subunits (β$_A$ or β$_B$), which combine to form activin A (β$_A$-β$_A$), activin B (β$_B$-β$_B$), or activin AB (β$_A$-β$_B$). Activin was discovered for its ability to regulate follicle-stimulating hormone (FSH) production by pituitary cells. Recently, the expressions of activin β$_A$ were found in many tissues throughout the body (Meunier et al., 1998; Tuuri et al., 1994), and activin is reported to act on many cell types, regulating hormone production in placental cell cultures (Petraglia et al., 1989), including differentiation in erythroblasts (Eto et al., 1987) and osteoblasts (Ogawa et al., 1992), inhibiting proliferation of gonadal cell lines (González-Manchón and Vale, 1989), endothelial cells (McCarthy and Bicknell, 1993), lung epithelial cells (Cárcamo et al., 1994), and hepatocytes (Yasuda et al., 1993), and inhibiting the differentiation of myoblasts (Link and Nishi, 1997; Shiozuka et al., 1997). Activin β$_A$ is highly expressed in adipose tissue (Vejda et al., 2002), however, the effect of activin on the adipocyte differentiation has been still unknown.

Activin exerts its biological effects by interacting with four types of transmembrane receptors (type IA, IB, IIA, IIB) with protein serine/threonine kinase activity (Attisano et al., 1996). Activin binds directory to the type II

receptors, leading to recruitment and phosphorylation of the type I receptors by the kinase domain of type II receptors. Once phosphorylated, type I receptors exhibit kinase activity on Smad proteins, intracellular signal mediators. Smad2 and Smad3 are specific to the activin signaling and are phosphorylated by activated activin receptors on serine residues. Phosphorylation of Smad2 and Smad3 allows complex formation with Smad4, a common effector shared by different TGFß family pathways (Heldin et al., 1997). Once formed the Smad2 / Smad4 or Smad3/ Smad4 complex translocates into the nucleus to activate transcription of specific target genes (Attisano and Wrana, 2000; Massagué and Wotton, 2000).

The expression sites of activin receptor mRNAs is reported to coincide with or adjacent to the sites of activin ß subunits expression during the period of organogenesis (Feijen et al., 1994). In postnatal adipogenesis, Rebbapragada et al. (2003) reported that myostatin blocks preadipocyte differentiation by binding to activin receptor IIB, however, no one has yet identified the expression of activin receptors or smads in adipose tissue or adipocytes.

Follistatin

Follistatin was purified from follicular fluids as a binding protein of activin (Nakamura et al., 1990). Follistatin has been shown to reverse the effects of activin on pituitary FSH release (Robertson et al., 1987; Ueno et al., 1987). Several follistatin proteins (FS-315, FS-303, and FS-288), resulting from alternative splicing (Shimasaki et al., 1988) and proteolytic cleavage (Inouye et al., 1991) have been purified. Alternative splicing generates two different

mRNAs, which encode FS-315 and its carboxy-terminally truncated homologue FS-288. Proteolytic cleavage converts FS-315 into FS-303, which is the major protein in follistatin preparations obtained from follicular fluid (Inouye et al., 1991). The affinities of the different follistatin proteins for activin A as determined by polyethylene glycol precipitation are essentially similar, whereas the affinity of FS-288 for heparan sulfate side chains of proteoglycans is much higher than that of FS-315 and FS-303 (Sugino et al., 1993). This can explain the higher potency of FS-288 in inhibiting FSH release (Inouye et al., 1991). Furthermore, FS-288 seems to have a higher affinity for an immobilized activin affinity column than FS-315 (Sumitomo et al., 1995), which can also explain the higher potency of FS-288.

Recently, myostatin is also reported to be captured by follistatin, which prevents myostatin binding to activin receptor IIB (Lee and McPherron, 2001). The studies on gene deletion (Matzuk et al., 1995) and overexpression (Lee and McPherron, 2001) of follistatin demonstrated that follistatin neutralizes the inhibitory effect of its sensitive ligands on muscle development, and myostatin is considered to be an obvious candidate for the sensitive ligand.

Follistatin mRNA and/or protein have been shown to be produced in many of the same tissues that produce activin (Shimasaki et al., 1988; Michel et al., 1990; DePaolo et al., 1991) including muscle and adipose tissue. These results suggest that follistatin can interact with activin and/or myostatin in several tissues.

Chapter 3

Effects of the conditioned medium from C_2C_{12} myocyte

on the differentiation of 3T3-L1 preadipocyte

Introduction

Beef marbling is characterized by adipose tissue deposition within skeletal muscle in cattle and is one of the important meat quality traits that influences juiciness and flavor of meat and contributes directly to the value of beef on especially Japanese markets. Japanese black cattle (Wagyu) is well known for its ability to produce high marbling (Lunt et al., 1993, Zenbayashi, 1994), and systemic fat metabolism does not necessarily reflect the fat deposition in the muscle in this breed (Mukai et al., 1995; Yang et al., 1985). Hood and Allen (1973) and Cianzio et al. (1985) reported that the development of beef marbling was closely associated with an increase in adipocyte number within muscle, suggesting that the proliferation and differentiation of adipose precursor cells could occur within muscle during the formation of beef marbling. As intramuscular preadipocytes are surrounded by mature skeletal muscle myofibers, it is predicted that paracrine factors secreted from muscle fibers may regulate the differentiation of preadipocyte via intercellular interactions.

Muscle fibers secrete various factors, such as fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), insulin-like growth factors (IGFs), hepatocyte growth factor (HGF), and

interleukin-6 (IL-6) (Chargé et al., 2004). These cytokines are known to regulate the proliferation and differentiation of preadipocytes, as well as of myoblasts (Chargé et al., 2004).

Myostatin, which is the member of TGF-β superfamily and is essential for proper regulation of skeletal muscle mass, is also known to be expressed mainly in skeletal muscle (Lee and McPherron, 1999). Furthermore, myostatin is reported to inhibit the differentiation of mouse preadipocytes (Kim et al., 2001).

Follistain is a secretory protein and it binds to activin, another member of TGF-β superfamily, and results in the prevention of activin from binding to the own receptors (Schneyer et al., 2003). Follistatin is also reported to capture myostatin, which prevents myostatin binding to activin receptors (Lee and McPherron, 2001).

To evaluate the effect of the paracrine factors from muscle cells on the differentiation of preadipocytes, the author used C_2C_{12} myoblast and 3T3-L1 preadipocyte cell lines, which are both well characterized and widely studied cell models for myoblast or preadipocyte differentiation. The conditioned medium from C_2C_{12} myocytes which contains paracrine factors secreted from C_2C_{12} cells was added to the medium of 3T3-L1 cell culture. Furthermore, the author hypothesized that the conditioned medium may contains myostatin, and investigated the effect of the co-treatment of the conditioned medium and follistatin on the differentiation of preadipocyte.

Materials and methods

C_2C_{12} cell culture and preparation of the conditioned medium

C_2C_{12} myoblasts, obtained from RIKEN Cell Bank (Tsukuba, Japan), were grown in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) with 10% fetal bovine serum (FBS) (Trace Biosciences, Melbourne, Australia), 100 U/ml penicillin, and 100 µg/ml streptomycin (each from Wako Chemicals, Osaka, Japan) at 37 °C under a humidified 5% CO_2 atmosphere. Two days after C_2C_{12} cells reached confluence (day 0), myogenesis was induced by changing the medium to serum free DMEM, and the medium was changed every second day. Conditioned medium (CM) was prepared from the medium collected on day 10 when most of the myoblasts were fused into myotubes. Part of the CM was ultrafiltrated in microconcentrators with 3,000 MW cut-off (Centriprep YM-3, Millipore, MA, USA) by centrifuging at 3,000 rpm at 4°C until 90% of the medium was filtrated. The nonpercolated medium was collected as the high molecular weight fraction (HCM) and the filtrate was as the low molecular weight fraction (LCM), and stored at -20°C.

3T3-L1 cell culture

3T3-L1 preadipocytes (Dainihon-seiyaku, Osaka, Japan), were subcultured in DMEM with 5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C under a humidified 5% CO_2 atmosphere. Adipogenesis was induced by the adipogenic agents (0.5 mM 3-isobutyl-1-methylxanthine, 0.25 µM dexamethasone (each from Sigma, MO, USA), and 10 µg/ml insulin (Wako

Chemicals)) in DMEM containing 5% FBS for 2 days after 3T3-L1 cells reached confluence (from day 0 to day 2). Then, the medium was replaced with DMEM containing 5% FBS and 5 μg/ml insulin, and was changed every second day. Thirty percent of the medium was replaced with each conditioned medium throughout the differentiation period (from day 0 to day 8), in the early phase of differentiation (from day 0 to day 2) or in the late phase of differentiation (from day 2 to day 8). Recombinant human follistatin (Genzyme Techne, MN, USA) was dissolved in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (Sigma) and added to the medium containing 30% HCM at the concentrations of 100, 300, 500 ng/ml in the early phase of differentiation (from day 0 to day 2).

Analysis of glycerol-3-phosphate dehydrogenase activity

Cells were carefully washed twice with ice-cold PBS on 8 day of differentiation period, and lysed in 25 mM Tris/1 mM EDTA, pH 7.5 for measurement of glycerol-3-phosphate dehydrogenase (GPDH) specific activity. GPDH activity was determined according to the procedure of Wise and Green (1979). Protein concentration was measured with the method of Lowry et al. (1951), and one unit of enzyme activity was defined as the amount of protein required for the use of 1 nmol NADH per min per mg protein.

Statistics

Data were expressed as means ± SE, and were statistically analyzed using student's t-test. Statistical significance was set at $P<0.05$.

Effect of the conditioned medium from C_2C_{12} myocyte on the differentiation of 3T3-L1 preadipocyte

To investigate the effect of the paracrine factors secreted from muscle cells on the differentiation of preadipocytes, 3T3-L1 cells were treated with the conditioned medium (CM, HCM, LCM) collected from almost fully differentiated C_2C_{12} cells (C_2C_{12} myocytes). LCM treatment did not affect GPDH activity regardless of the treatment period (Fig. 3-1). CM or HCM treatment throughout the differentiation period or in the early phase of differentiation significantly reduced the GPDH activity (Fig. 3-1). GPDH activity was also decreased by the CM treatment in the late phase, however, HCM treatment in the late phase did not affect GPDH activity (Fig. 3-1).

Effect of the co-treatment of high molecular weight fraction of the conditioned medium and follistatin on the differentiation of 3T3-L1 preadipocyte

To examine the possibility that myostatin and/or activin in the conditioned medium affect the differentiation of 3T3-L1 preadipocytes, the cells were treated with HCM and follistatin in the early phase of differentiation. HCM suppressed GPDH activity when the cells were treated in the early phase of differentiation (Fig. 3-2). The simultaneous treatment with follistatin reversed the suppressive effect of HCM in a dose dependent manner (Fig. 3-2).
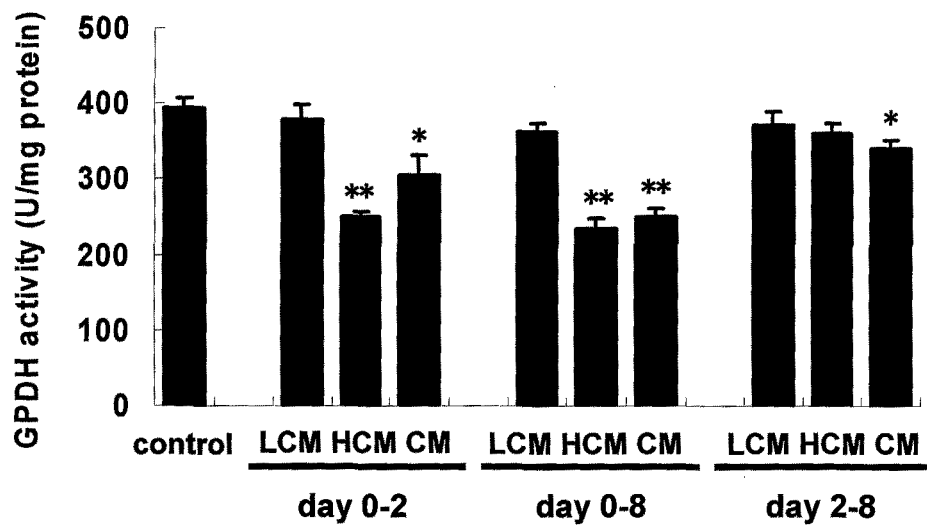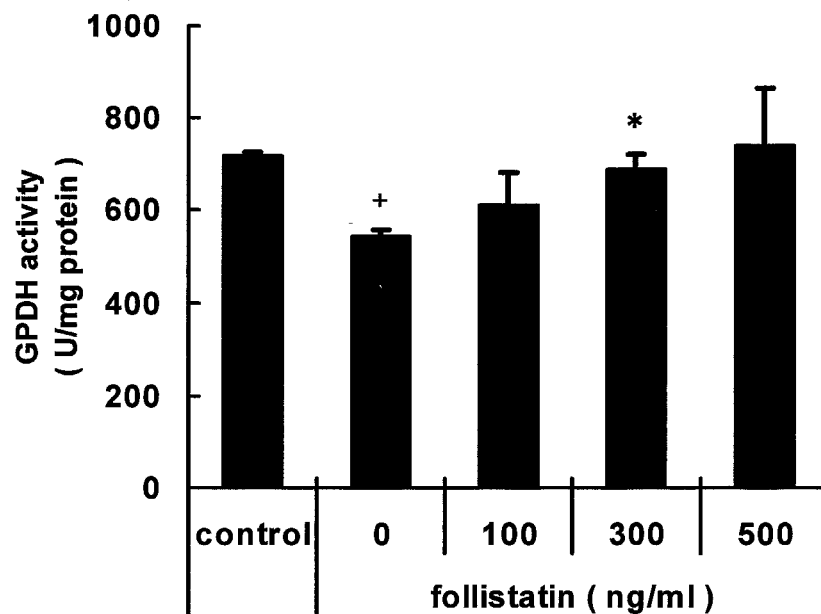
Fig.3-1. Effect of the conditioned medium from C_2C_{12} myocyte on the differentiation of 3T3-L1 preadipocyte.

Conditioned medium (CM) was collected from the C_2C_{12} cells on day 10. The collected medium was ultrafiltrated in microconcentrators with 3000 MW cut-off (Centriprep YM-3, Millipore, MA, USA). The nonpercolated medium was collected as the high molecular weight fraction (HCM) and the filtrate was as the low molecular weight fraction (LCM). 3T3-L1 cells were treated with LCM, HCM, or CM by replacing 30% of the medium in the early phase of differentiation (from day 0 to day 2), throughout the differentiation period (from day 0 to day 8), or in the late phase of differentiation (from day 2 to day 8). Control cells were cultured in the medium treated without conditioned medium. GPDH activity per mg protein was determined. Values are means ± SE for three replicated cultures. *P<0.05, **P<0.01 vs control.

Fig. 3-2. Effect of the high molecular weight fraction of the conditioned medium and follistatin on the differentiation of 3T3-L1 preadipocyte.

3T3-L1 cells were treated with high molecular weight fraction of the conditioned medium (HCM) by replacing 30% of the medium, and follistatin in various concentrations in the early phase of differentiation (from day 0 to day 2). Control cells were cultured with neither HCM nor follistatin. Cells were harvested at the end of culture (day 8) and GPDH activity was determined as described in the Fig. 3-1. Values are means ± SE for three replicated cultures. [+]P<0.05 vs control culture treated neither HCM nor follistatin; [*]P<0.05 vs culture treated with HCM alone.

Discussion

In the present study, the treatment with CM or HCM throughout the differentiation period significantly reduced GPDH activity, a terminal differentiation marker. The treatment with CM or HCM also decreased GPDH activity when the cells were treated in the early phase of differentiation. However, the treatment with HCM in the late phase did not affect GPDH activity and only CM slightly decreased the activity. These results suggest that the high molecular weight factors secreted from C_2C_{12} myocytes mostly suppressed the preadipocyte differentiation in the early phase that is the step of commitment to differentiate in 3T3-L1 cells (Gregoire et al., 1998).

The 3T3-L1 cell line is the most well-characterized and reliable model for studying preadipocyte differentiation. A study using 3T3-L1 preadipocyte showed that CCAAT/enhancer binding protein (C/EBP) β was expressed immediately (within 2-4 hours) by induction of differentiation, reached maximal level within 4 hours, and begins to disappear 2 days after initiation of differentiation (Lane et al., 1999). C/EBPβ transcriptionally activates peroxisome proliferator-activated receptor (PPAR) γ expression during the induction of differentiation, which reaches maximal level on day 3 (Chawla et al., 1994). The expression of C/EBPα is also activated by C/EBPβ but the level of C/EBPα is faint during the induction and reaches maximal level by 5 days after initiation of differentiation (Christy et al., 1991). Thereafter, PPARγ and C/EBPα upregulate each other to maintain their expression (Mandrup and Lane, 1997) despite the reduction of C/EBPβ level. PPARγ and C/EBPα coordinately activate the

expression of adipocyte-specific genes (Gregoire et al., 1998).

Myostatin, which is essential for proper regulation of skeletal muscle mass, is known to be expressed mainly in skeletal muscle. Artaza et al. (2002) reported that the expression of myostatin mRNA was found in C_2C_{12} myotubes not in C_2C_{12} myoblasts, and that myostatin was also secreted into the medium of C_2C_{12} myotube culture. Furthermore, myostatin is reported to inhibit the differentiation of 3T3-L1 preadipocytes via affecting the expression of PPARγ and C/EBPα, but not C/EBPβ (Kim et al., 2001).

In the present study, HCM treatment inhibited the preadipocyte differentiation in the early phase when the expressions of PPARγ and C/EBPα are induced in 3T3-L1 preadipocytes. Therefore, it is predicted that HCM inhibited the differentiation of 3T3-L1 preadipocyte by suppressing the expression of PPARγ and C/EBPα, although the mechanism was not investigated in this study.

Based on these reports and results in the present study, myostatin was considered to be a candidate for the effective factor in the HCM that inhibited the differentiation of 3T3-L1 preadipocyte. To examine this possibility, the differentiating 3T3-L1 cells were co-treated with the HCM and follistatin, which binds to myostatin and activin and inhibits their action. Follistatin reversed the inhibitory effect of HCM on the differentiation of 3T3-L1 preadipocytes in a dose dependent manner, which suggested that myostatin and/or activin in the HCM possibly inhibited the preadipocyte differentiation.

The expression of activin is widely distributed in gonadal and non-gonadal tissues including muscle (Tuuri et al., 1994) and fat (Schneider et al., 2000; Vejda

et al., 2002). Furthermore, C_2C_{12} myoblasts and myocytes in the present study also expressed activin mRNA (data not shown). Activin is reported to act on many cell types, including placental cells (Petraglia et al., 1989), erythroblasts (Eto et al., 1987), osteoblasts (Ogawa et al., 1992), endothelial cells (McCarthy and Bicknell, 1993), and myoblasts (Link and Nishi, 1997; Shiozuka et al., 1997). However, effect of activin on the differentiation of preadipocytes has been still unknown. Moreover, the expression of activin receptors, which mediate the action of activin (Mathews, 1994; Piek et al., 1999) and myostatin (Lee and McPherron, 2001; Massagué and Chen, 2000), has not been investigated in adipose tissue. Further study is needed to determine the effect of activin on the differentiation of preadipocytes.

In summary, these results presented here showed that conditioned medium from C_2C_{12} myocytes inhibited the preadipocytes differentiation in the early phase, and this inhibitory effect was possibly caused by myostatin and/or activin secreted from C_2C_{12} myocytes.

Chapter 4

Effects of myostatin on the differentiation of bovine preadipocyte

Introduction

CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor (PPAR) γ have shown to be critical transcription factors for preadipocyte differentiation (Gregoire et al., 1998; Rosen et al., 2000). Studies using 3T3-L1 preadipocytes indicate that C/EBPβ is highly expressed after the treatment with adipogenic agents (Lane et al., 1999). C/EBPβ activates the expression of PPARγ and C/EBPα mRNAs that coordinately activate the transcription of adipocyte-specific genes (Rosen et al., 2000). On the other hand, these 3 transcription factors were highly expressed before inducing the differentiation of preadipocytes in the primary cultures of porcine stromal-vascular (SV) cells derived from adipose tissue (Ding et al., 1999).

In Chapter 3, the author revealed that the high molecular weight fraction of the conditioned medium from C_2C_{12} cells suppressed the differentiation of 3T3-L1 preadipocytes, and this inhibitory effect of the conditioned medium was reversed by follistatin which is known to bind to myostatin (Lee and McPherron, 2001) and/or activin (Schneyer et al., 2003). These results suggest that myostatin and/or activin in the conditioned medium possibly affected the preadipocyte differentiation.

Myostatin (growth differentiation factor-8, GDF-8) is a member of the

transforming growth factor-β (TGF-β) superfamily, and a key critical regulator of skeletal muscle development (McPherron et al., 1997). In cattle, defective mutation of myostatin gene increased skeletal muscle, which is known as double-muscling (McPherron and Lee, 1997). The expression of myostatin mRNA is found primarily in skeletal muscle, but it is also detected in the adipose tissue (McPherron et al., 1997). Myostatin can bind the activin type II receptors, which leads to the intracellular signal transduction (Lee and McPherron, 2001; Massagué and Chen, 2000). Myostatin was reported to suppress the induction of PPARγ and C/EBPα in 3T3-L1 cells after the initiation of differentiation, which interfered with preadipocyte differentiation (Kim et al., 2001). However, the effect of myostatin on the differentiation of bovine preadipocytes has been unknown. Furthermore, the expression patterns of the critical transcription factors of bovine preadipocyte differentiation have not well studied.

The purpose in this chapter was to investigate the expression of these transcription factor mRNAs during preadipocyte differentiation in stromal vascular (SV) cells derived from bovine adipose tissue, and to examine the effect of myostatin on the expression of these transcription factor mRNAs and on the terminal differentiation of bovine preadipocytes. Furthermore, the author investigated whether follistatin interferes with myostatin action during bovine preadipocyte differentiation when the cells were treated with myostatin and follistatin.

Materials and methods

Preparation of stromal-vascular cells from bovine adipose tissue

Perirenal adipose tissues were collected from 28-32 months old Japanese black steers at a local slaughter house and transported to the laboratory in sterile Hanks balanced salt solution (HBSS) containing 100 U/ml penicillin, 100 µg/ml streptomycin (each from Wako Chemicals), and 250 ng/ml amphotericin B (Invitrogen, CA, USA). The adipose tissues were digested in HBSS added with 1 mg/ml Type I collagenase (Sigma) for 1 h at 37 °C with shaking at 170 cycles/min. The cell suspension was filtrated through a 250 µm nylon mesh filter to remove undigested tissue fragments and debris. The filtrate was then centrifuged at 1,500 rpm for 5 min. Floating adipocytes and digestion medium were removed by decantation. The pellet consists of SV cells containing preadipocytes. The SV cells were washed twice with the growth medium, i.e., DMEM (Nissui) containing 5% FBS (Trace Biosciences), 100 µM ascorbic acid phosphate magnesium, 100 U/ml penicillin, 100 µg/ml streptomycin (Wako Chemicals). The SV cells were then resuspended with the growth medium containing 10% dimethyl sulfoxide and stored in liquid nitrogen.

Cell culture

The SV cells were seeded on 12-well (22 mm diameter) culture plates (Corning, NY, USA) at a density of 1×10^4 cells/cm^2 and incubated in the growth medium at 37 °C under a humidified 5% CO_2 atmosphere. After reaching confluence, preadipocyte differentiation was induced by the adipogenic agents consisting of

0.5 mM 3-isobutyl-1-methylxanthine, 0.25 µM dexamethasone (each from Sigma), 2.5 µg/ml insulin (Wako Chemicals) and 5 µM troglitasone (Sankyo, Tokyo, Japan) for 2 days (from day 0 to day 2). The medium was replaced with DMEM containing 5% FBS, 2.5 µg/ml insulin, and 5 µM troglitasone, and was changed every second day. Recombinant human myostatin (PeproTech EC, London, UK) and recombinant human follistatin (Genzyme Techne) was dissolved in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (Sigma) and added to the medium in the concentrations as shown in each figure during the early phase of differentiation (from day 0 to day 2) or throughout the differentiation period (from day 0 to day 8).

Glycerol-3-phosphate dehydrogenase activity

Cells were carefully washed twice with ice-cold PBS on day 8, and lysed in 25 mM Tris/ 1 mM EDTA, pH 7.5 by sonication for measurement of GPDH specific activity. GPDH activity was determined according to the method shown in Chapter 3.

Oil Red O staining

Cultures were washed twice with PBS on day 0 and day 8, fixed with 10% formalin in PBS, stained with 0.5% Oil Red O and photographed.

RNA isolation

Total RNA was extracted from the SV cells using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. The extracted RNA was dissolved in

diethyl pyrocarbonate-treated water and total RNA concentration was determined spectrophotometrically at 260 nm.

Detection of activin receptor mRNAs in bovine preadipocyte

The expression of activin receptor mRNAs encoding bovine type I (ActRI) and type II (ActRIIA and ActRIIB) receptors on day 0 were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Single-strand cDNA was synthesized using a TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Shiga, Japan) according to the manufacture's protocol. The PCR were conducted using Platinum PCR Super Mix (Invitrogen). The primer sets for each receptor were shown in Table 1. The amplification parameters consisted of denaturation at 94 °C for 30 s, annealing at the temperature (Table 4) for 30 s, and extension at 72 °C for 1 min, for 35 cycles. The subcloning and sequencing confirmed the PCR products to be the expected fragments of bovine activin receptors. The products were separated on 2% agarose gel and visualized with ethidium bromide. To avoid false-positive results from contamination by genomic DNA, samples with or without treatment of reverse transcriptase were prepared, and as a negative control, RT-PCR without RNA samples was also carried out.

Northern blot analysis for the expression of aP2 mRNA

The author analyzed the expression of aP2 mRNA on day 0 and day 8. cDNA fragment of bovine adipocyte fatty acid-binding protein (aP2) gene was amplified using specific primers (Table 4) and inserted into the pCR II TOPO plasmid vector (Invitrogen). The template was linearized with Not I (TOYOBO, Tokyo,

Japan) and then used in DIG RNA labeling Kit (Roche Diagnostics, Mannheim, Germany) to synthesize the digoxigenin (DIG) -dUTP-labeled RNA antisense probe. RNA sample (5 µg) was separated on 1% agarose gels containing 6.7% formaldehyde and transferred to a Hybond N+ membrane (Amersham Biosciences, NJ, USA). The membrane was hybridized with DIG-labeled RNA probe. The band corresponding to aP2 mRNA was detected using DIG Luminescant Detection Kit for Nucleic Acids (Roche Diagnostics).

Semi-quantitative RT-PCR for the determination of C/EBP and PPARγ mRNAs

The expression of C/EBP and PPARγ mRNAs were assessed by semi-quantitative RT-PCR. Single-strand cDNA was synthesized using a TaKaRa RNA PCR Kit (AMV) Ver.3.0 according to the manufacture's protocol. The thermal amplifications of C/EBP fragments were conducted using TaKaRa LA Taq with GC Buffer. PPARγ and 18S rRNA fragments were amplified by using Platinum PCR Super Mix (Invitrogen). The primer sets were shown in Table 1. PCR conditions were optimized for detection within a linear range. The amplification parameters consisted of denaturation at 94 °C for 30 s, annealing at the temperature (Table 4) for 30 s, and extension at 72 °C for 1 min, for the appropriate number of cycles; 35 cycles for the detection of C/EBPß, 27 cycles for PPARγ, 30 cycles for C/EBPα, and 18 cycles for 18S rRNA. The subcloning and sequencing confirmed the PCR products to be the expected fragments of bovine C/EBP, PPARγ, and 18S rRNA. The level of 18S rRNA was adopted as the internal standards for the determination of targeted mRNA levels.

Statistics

Data were expressed as means ± SE, and were statistically analyzed using student's t-test. Statistical significance was set at P<0.05.

Table 4. Sequences of the primer sets

Primers	Forward	Reverse	Annealing temp, °C	Fragment size, bp	GenBank accession number
bActRI	AAATGGGATCGCTGTACGACT	GGCCAGGGAGGTTAATGTCG	56	554	U58095
bActRIIA	TTGGGAAAGAGATAGAACGAATCG	GCTTAGGAGTTACTGGATTCGAC	56	293	U43208
bActRIIB	GGCCCAGAAGTCACGTACGA	GACAGCCACGAAGTCGTTCA	63	288	M84120
bC/EBPα	TCGCCATGCCGGGAGGACT	GTGTGCGATCTGGAACTGC	63	654	D82984
bC/EBPβ	GCCGAGTACGGCTACGTGAG	CCGCCGGATCTTGTACTCGTC	58	441	D82985
bPPARγ	CAGCATTTCCACTCCGCACTA	TCTGGGTTCAGTTGGTCGATA	56	492	AY179866
baP2	GTGTGATGCATTTGTAGGT	CTGGTGGCAGTGACACCAT	56	378	X89244
b18S rRNA	GGAATCAGGGTTCGATTCCG	GGACACTCAGCTAAGAGCATCGA	58	370	AF176811

Expression of activin receptor mRNAs

Expression of mRNAs coding bovine type I activin receptor (ActRI) and type II activin receptors (ActRIIA and ActRIIB) were examined by RT-PCR. These receptor mRNA expressions were detected in the postconfluent bovine SV cells (Fig. 4-1). In the absence of reverse transcriptase or RNA samples during the synthesis of cDNA, no bands were detected (data not shown).

Differentiation of bovine preadipocyte

First, the author investigated the adipocyte-specific markers of mature adipocytes in the SV cells prior to the induction of differentiation (day 0). Neither Oil Red O staining-positive cells nor aP2 mRNA expression in the SV cells could be detected on day 0 (data not shown). Oil Red O staining at the end of culture showed that many control cells had lipid droplets (Fig. 4-2A). The highest dose of myostatin (300 ng/ml) inhibited lipid accumulation (Fig. 4-2A), GPDH activity (Fig. 4-2B), and the level of aP2 mRNA (Fig. 4-2C) when the cells were treated with myostatin in the early phase of differentiation. On the other hand, the lower dose of myostatin was sufficient to decrease lipid accumulation (Fig. 4-3A), GPDH activity (Fig. 4-3B), and aP2 mRNA level (Fig. 4-3C) in the cells treated with myostatin throughout the differentiation period.

Expression of C/EBP and PPARγ mRNAs

C/EBPβ mRNA in the control cells was stably expressed throughout the

differentiation period (Fig. 4-4A). Myostatin treatment throughout the differentiation period did not affect the expression of C/EBPβ mRNA until day 4, however, myostatin significantly decreased C/EBPβ mRNA level thereafter (Fig. 4-4A). The control cells expressed PPARγ mRNA on day 0, then its expression moderately increased, and it reached almost maximal level on day 2 (Fig. 4-4B). Myostatin suppressed the increase in PPARγ mRNA expression during the induction of differentiation and PPARγ mRNA level decreased thereafter (Fig. 4-4B). The control cells faintly expressed C/EBPα mRNA on day 0, its expression started to increase on day 2, and the large increase was observed between day 4 and day 6 (Fig. 4-4C). Myostatin strongly suppressed the induction of C/EBPα mRNA (Fig. 4-4C).

Myostatin and follistatin treatments

The author investigated whether follistatin affected the suppressive action of myostatin on the differentiation of bovine preadipocytes. Myostatin suppressed GPDH activity when the cells were treated with myostatin in the early phase of differentiation (Fig. 4-5). The simultaneous treatment with follistatin reversed the suppressive effect of myostatin in a dose dependent manner (Fig. 4-5).
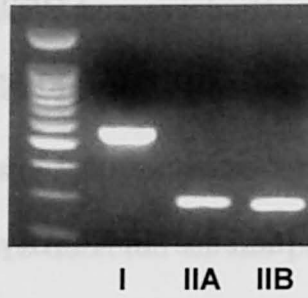
Fig. 4-1. Expression of activin receptor mRNAs in bovine stromal-vascular (SV) cells. Post confluent (day 0) bovine SV cells were harvested and RT-PCR was performed using primer sets for bActRI (I), bActRIIA (IIA), or bActRIIB (IIB). The products were separated on 2% agarose gel and visualized with ethidium bromide.

A

0   100   300

myostatin (ng/ml)

B

0   100   300

myostatin (ng/ml)

C

aP2

28S
18S

0   100   300
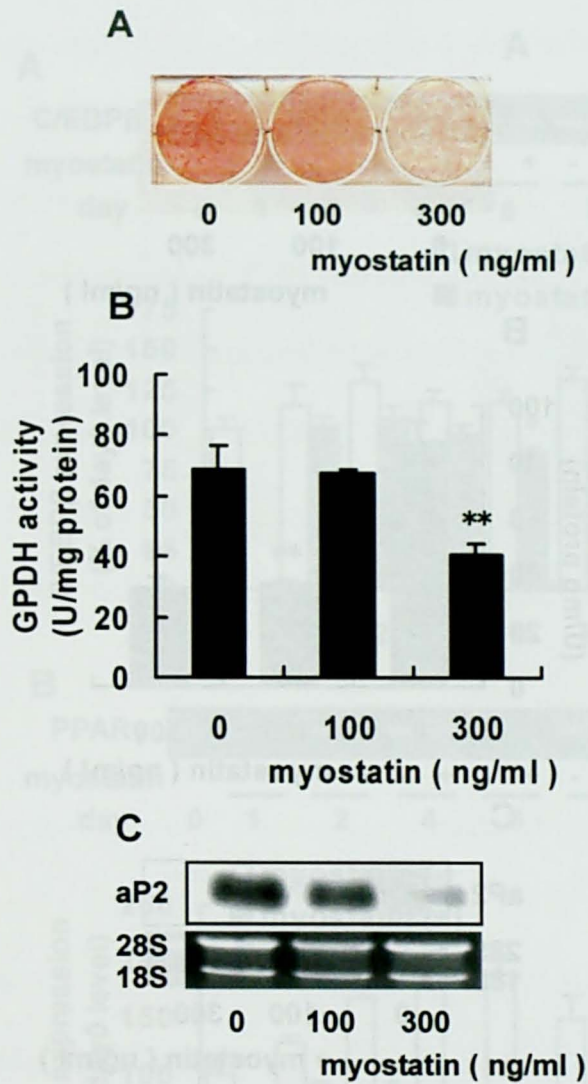
myostatin (ng/ml)

Fig. 4-2. Effect of myostatin treatment in the early phase on bovine preadipocyte differentiation.

Bovine SV cells were treated with myostatin for 2 days after confluence (from day 0 to day 2) and cells were harvested at the end of culture (day 8). Intracellular lipid was stained with Oil Red O (A). GPDH activity per mg protein was determined (B). The expression of aP2 mRNA was measured by Northern blot analysis. Ethidium bromide staining demonstrates the RNA loading (C). Values are means ± SE for 4 replicated cultures. **P<0.01 vs culture treated without myostatin.

A

0   100   300
myostatin (ng/ml)

B

0   100   300
myostatin (ng/ml)

C

aP2

28S
18S

0   100   300
myostatin (ng/ml)
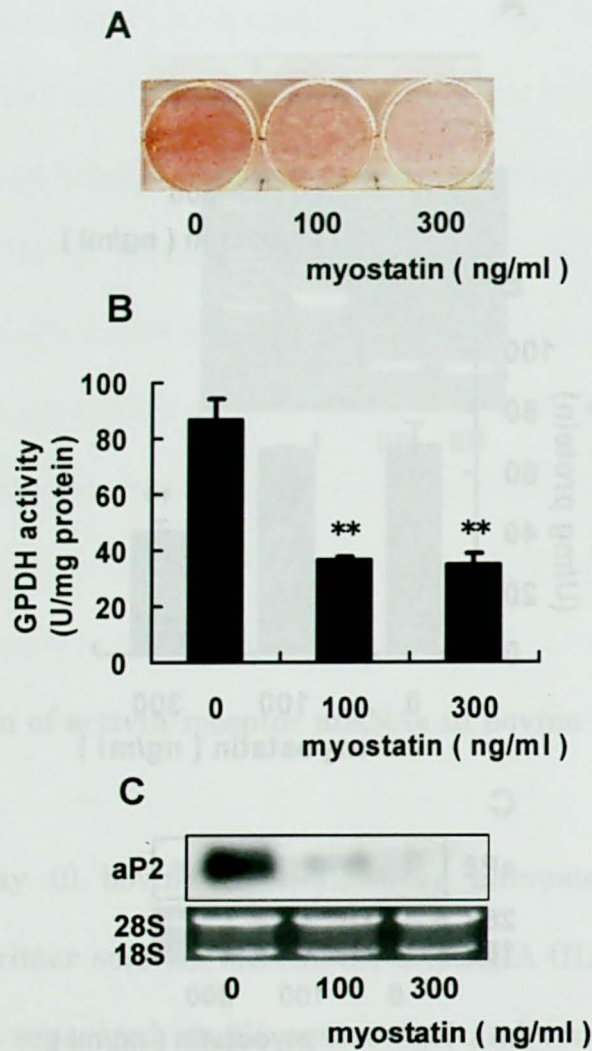
Fig. 4-3. Effect of myostatin treatment throughout the differentiation period on bovine preadipocyte differentiation.

Bovine SV cells were treated with myostatin for 8 days after confluence (from day 0 to day 8). Intracellular lipid (A), GPDH activity (B), and Northern blot analysis for the expressions of aP2 mRNA (C) were determined as described in the Fig. 4-2 legend. Values are means ± SE for 4 replicated cultures. **P<0.01 vs culture treated without myostatin.

A

C/EBPβ

myostatin - + - + - + - + - +
day 0 1 2 4 6 8

☐ myostatin (-)
■ myostatin (+)

C/EBPβ expression
(% of day0 level)

175
150
125
100
75
50
25
0

0 1 2 4 6 8 (day)

B

PPARγ

myostatin - + - + - + - + - +
day 0 1 2 4 6 8

☐ myostatin (-)
■ myostatin (+)

PPARγ expression
(% of day0 level)

250
200
150
100
50
0

0 1 2 4 6 8 (day)

C

C/EBPα

myostatin - + - + - + - + - +
day 0 1 2 4 6 8

C/EBPα expression
(% of day0 level)

1200
1000
800
600
400
200
0

☐ myostatin (-)
■ myostatin (+)

0 1 2 4 6 8 (day)
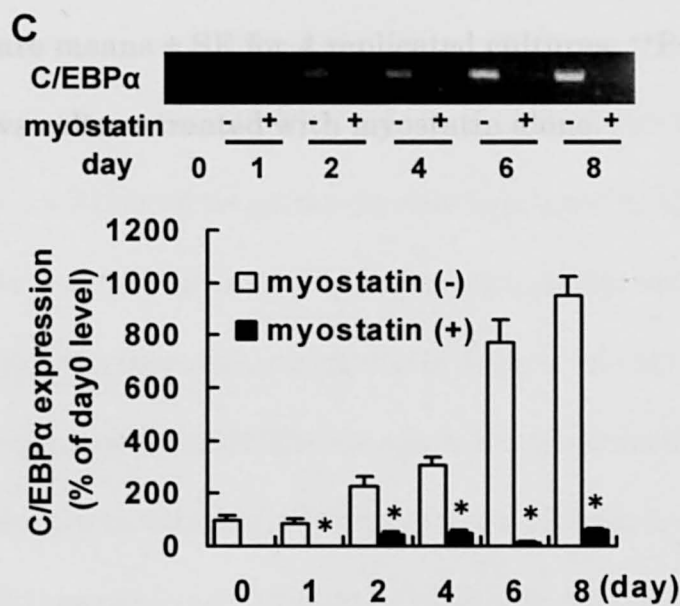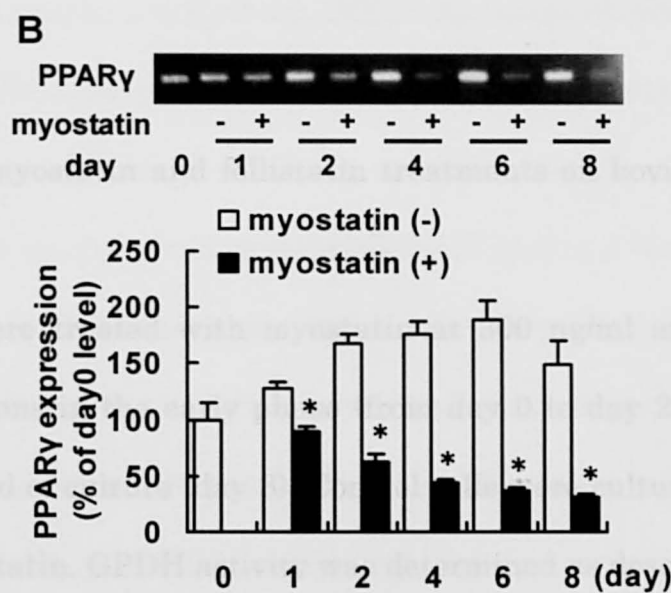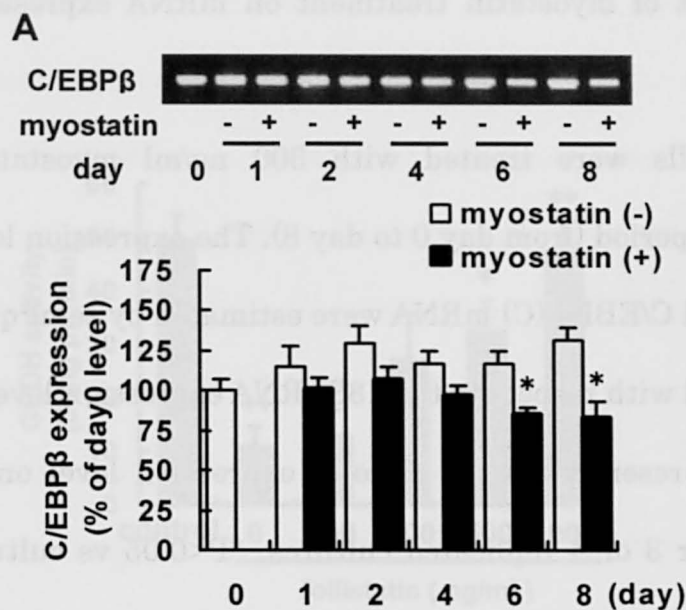
Fig. 4-4. Effect of myostatin treatment on mRNA expressions of C/EBP and PPARγ.

Bovine SV cells were treated with 300 ng/ml myostatin throughout the differentiation period (from day 0 to day 8). The expression levels of C/EBPβ (A), PPARγ (B), and C/EBPα (C) mRNA were estimated by semi-quantitative RT-PCR and normalized with respect to the 18S rRNA expression level. The relative gene expression is presented as the ratio of expression level on day 0. Values are means ± SE for 3 or 4 replicated cultures. *P<0.05 vs culture treated without myostatin.

GPDH activity
(U/mg protein)

60
50
40
30
20
10
0

++   ++   *   **

control   0   100   300   500   1000
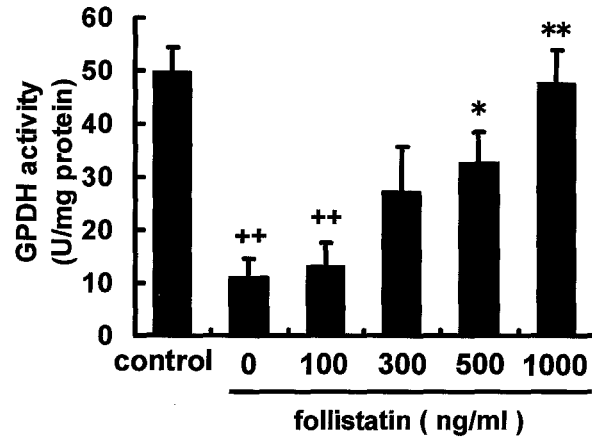
follistatin (ng/ml)

Fig. 4-5. Effect of myostatin and follistatin treatments on bovine preadipocyte

differentiation.

Bovine SV cells were treated with myostatin at 300 ng/ml and follistatin in

various concentrations in the early phase (from day 0 to day 2) and cells were

harvested at the end of culture (day 8). Control cells were cultured with neither

myostatin nor follistatin. GPDH activity was determined as described in the Fig.

4-2 legend. Values are means ± SE for 4 replicated cultures. ++P<0.01 vs control;

*P<0.05, **P<0.01 vs culture treated with myostatin alone.

Discussion

Myostatin secreted from muscular cells regulates their cellular function via activin receptors, i.e., myostatin binds to cell surface activin type II receptors, mainly to ActRIIB (Lee and McPherron, 2001), and to form a heteromeric complex with activin type I receptor (ActRI) (Massagué and Chen, 2000), which transduces to the common intracellular signals of myostatin and activin. The present study demonstrated mRNA expressions of type I activin receptor (ActRI) and type II activin receptors (ActRIIA and ActRIIB) in post confluent bovine SV cells derived from adipose tissue. These results suggest that bovine SV cells can respond to myostatin.

In the SV cells on day 0, adipocyte-specific markers such as Oil Red O staining-positive cells and aP2 mRNA expression could not be detected. Additionally, the expression of C/EBPα mRNA was faint on day 0 but markedly increased during preadipocyte differentiation. These results indicated that the SV cells in the present study did not contain significant number of adipocytes prior to the induction of differentiation (day 0).

Myostatin treatment throughout the differentiation period reduced GPDH activity, aP2 mRNA level, and accumulation of lipid. However, its treatment in the early phase of differentiation only modestly reduced these differentiation markers. These results suggest that myostatin continuously suppresses bovine preadipocyte differentiation throughout the differentiation period.

The 3T3-L1 cell line is the most well-characterized and reliable model for studying preadipocyte differentiation. A study using 3T3-L1 preadipocyte

showed that C/EBPβ was expressed immediately (within 2-4 hours) by induction

of differentiation, reached maximal level within 4 hours, and begins to disappear

2 days after initiation of differentiation (Lane et al., 1999). C/EBPβ

transcriptionally activates PPARγ expression during the induction of

differentiation, which reaches maximal level on day 3 (Chawla et al., 1994). The

expression of C/EBPα is also activated by C/EBPβ but the level of C/EBPα is faint

during the induction and reaches maximal level by 5 days after initiation of

differentiation (Christy et al., 1991). Thereafter, PPARγ and C/EBPα upregulate

each other to maintain their expression (Mandrup and Lane, 1997) despite the

reduction of C/EBPβ level. PPARγ and C/EBPα coordinately activate the

expression of adipocyte-specific genes (Gregoire et al., 1998).

The present experiment showed that C/EBPβ mRNA in the bovine control cells

was stably expressed throughout the differentiation period. Additionally, the

relatively high expression of PPARγ was observed before the induction of

differentiation. These results are similar to porcine SV cell culture (McNeel et al.,

2000). On the other hand, C/EBPα mRNA level was extremely low before the

induction of differentiation in bovine SV cells, but porcine SV cells expressed

considerable C/EBPα before the induction (Ding et al., 1999; McNeel et al., 2000).

These results suggest that porcine preadipocytes reached at the most advanced

stage of differentiation, 3T3-L1 preadipocytes were most immature, and the

bovine preadipocytes in the present study reached an intermediate stage

between porcine preadipocytes and 3T3-L1 cells when the cells were confluent.

The present experiment indicated that myostatin suppressed the increase in

PPARγ mRNA expression and the induction of C/EBPα mRNA without affecting

the expression of C/EBPβ mRNA in the early phase of differentiation. Additionally, the expression of C/EBPβ mRNA was not affected by myostatin until day 4 but PPARγ mRNA level was decreased and C/EBPα mRNA level was continuously low in the bovine preadipocytes treated with myostatin. These results suggest that myostatin suppressed bovine preadipocyte differentiation via inhibiting the transcriptional cascade downstream of C/EBPβ mRNA expression. Kim et al. (2001) reported that myostatin reduced PPARγ and C/EBPα levels but did not affect C/EBPβ expression in 3T3-L1 preadipocytes. Activin A is reported to repress the transactivation functions of C/EBPβ in hepatocyte culture mediated by Smad3 interaction with the DNA binding domain of C/EBPβ (Zauberman et al., 2001). As mentioned above, myostatin is reported to regulate cellular function via activin receptors and the intracellular signals of activin (Rebbapragada et al., 2003). Therefore, myostatin possibly prevents bovine preadipocyte differentiation via Smad3-mediated impairment of the transactivation function of C/EBPβ. In the present study, the differentiation was more severely suppressed by the treatment with myostatin throughout the differentiation period than by the treatment in the early phase of differentiation. The expression of C/EBPβ begins to decline 2 days after the initiation of differentiation in 3T3-L1 cells (Lane et al., 1999), but bovine preadipocyte expressed C/EBPβ mRNA throughout the differentiation period. Additionally, myostatin reduced C/EBPβ mRNA level of bovine preadipocyte in the late phase of differentiation. The severe inhibition of differentiation induced by the treatment throughout the differentiation period probably result from the continuous suppression of C/EBPβ function and/or the reduction of C/EBPβ

mRNA expression in the late phase.

Systemic administration of myostatin to adult mice results in a cachexia-like syndrome that is associated with a profound loss of both muscle and fat (Zimmers et al., 2002). The reduction of fat accumulation is also observed in knock-out mice that lacks myostatin (Lin et al., 2002; McPherron and Lee, 2002) and in the double-muscled cattle that have mutations in the myostatin gene (Kobolak and Gocza, 2002). It is not clarified how myostatin regulates postnatal fat accumulation, however, unphysiological condition such as lack of myostatin may affect energy metabolism and prevent fat accumulation elsewhere in the body (McPherron and Lee, 2002). Another possibility is that myostatin differently affects adipogenesis in postnatal and fetal period. Artaza et al. (2005) recently reported that myostatin inhibited myogenesis and promoted adipogenesis in mesenchymal multipotent cells, and they suggested that myostatin promoted the differentiation of multipotent cells into the adipogenic lineage. Therefore, myostatin possibly stimulates the commitment of mesenchymal cells to preadipocytes but suppresses the differentiation of committed preadipocytes such as 3T3-L1 cells (Kim et al., 2001) and bovine preadipocytes in the present study. The commitment to preadipocytes might be reduced in myostatin-deleted animals during embryogenesis and the differentiation of committed preadipocytes might be suppressed by myostatin administration in postnatal life, which can explain the in vivo apparent discordance that either myostatin deletion (Kobolak and Gocza, 2002; Lin et al., 2002; McPherron and Lee, 2002) or myostatin administration (Zimmers et al., 2002) decreases adipogenesis.

Langley et al. (2002) reported that 8 μg/ml of myostatin was required for inhibiting the differentiation of both C_2C_{12} myoblasts and bovine myoblasts derived from semitendinosus muscle of fetuses. This effective dose of myostatin in myoblasts was much higher than the dose (100 ng/ml) affecting bovine preadipocyte differentiation in the present study. Thus, it is possible that myostatin produced in adipose tissue directly affects preadipocyte differentiation though its level is substantially lower than in skeletal muscle (McPherron et al., 1997). Additionally, myostatin secreted from skeletal muscle may affect the differentiation of committed preadipocytes surrounding muscle. Further study is needed to clarifying the physiological action of myostatin on adipogenesis in vivo. The present experiment indicated that follistatin reversed the inhibitory effect of myostatin on bovine preadipocyte differentiation. Follistatin is known to capture myostatin, which prevents myostatin binding to ActRIIB (Lee and McPherron, 2001). The studies on gene deletion (Matzuk et al., 1995) and overexpression (Lee and McPherron, 2001) of follistatin demonstrated that follistatin neutralizes the inhibitory effect of its sensitive ligands on muscle development, and myostatin is considered to be an obvious candidate for the ligand. Follistatin is widely distributed not only in gonadal tissues but also in other tissues including adipose tissue (Schneider et al., 2000). Therefore, it is predicted that the interaction between myostatin and follistatin also exists in adipose tissue. The physiological balance of myostatin and follistatin possibly regulates bovine preadipocyte differentiation as local factors.

The experiment in this chapter showed that myostatin inhibited bovine preadipocyte differentiation at the smaller dose than that inhibited bovine

myoblast differentiation (Langley et al., 2002). The inhibitory effect of myostatin

was mediated by the reduction of PPARγ and C/EBPα mRNA levels. Additionally,

follistatin affected bovine preadipocyte differentiation, which suggested that

follistatin modulated the action of myostatin on preadipocyte differentiation.

Chapter 5

The role of activin A on the preadipocyte differentiation

Section 1

Effects of activin A on the differentiation of bovine preadipocyte

Introduction

Activin A, a member of the transforming growth factor-β (TGF-β) superfamily, is homodimer of activin $β_A$ subunit. Recently, the expressions of activin $β_A$ (Meunier et al., 1988; Tuuri et al., 1994) and activin receptor mRNAs (Mathews, 1994) were found in many tissues throughout the body. Especially, activin $β_A$ was highly expressed in adipose tissue (Vejda et al., 2002).

CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor (PPAR) γ have shown to be critical transcription factors for preadipocyte differentiation (Gregoire et al., 1998; Rosen et al., 2000). Studies using 3T3-L1 preadipocytes indicate that C/EBPβ is highly expressed after the treatment with adipogenic agents (Lane et al., 1999). C/EBPβ activates the expression of PPARγ and C/EBPα mRNAs that coordinately activate the transcription of adipocyte-specific genes (Rosen et al., 2000).

In Chapter 4, the author investigated the expression patterns of the critical transcription factors in stromal vascular (SV) cells from bovine adipose tissue.

SV cells did not express adipogenic differentiation markers such as lipid accumulation and adipocyte fatty acid-binding protein (aP2) mRNA before the induction of differentiation. On the other hand, C/EBPβ mRNA was stably expressed throughout the differentiation period, and the expression of PPARγ was relatively high before the induction of differentiation. C/EBPα mRNA was only faintly expressed before the induction of differentiation and increased thereafter. These results suggested that bovine SV cells derived from adipose tissue contained the preadipocytes reaching at more advanced stage of differentiation than 3T3-L1 preadipocytes did.

Kim et al. (2001) reported that myostatin, which is also a member of TGF-β superfamily, inhibited the differentiation of 3T3-L1 preadipocytes by reducing the expression of PPARγ and C/EBPα but not of C/EBPβ. Despite the different expression patterns of the critical transcription factors in bovine preadipocytes, myostatin also inhibited bovine preadipocyte differentiation via affecting the transcriptional cascade downstream of C/EBPβ as demonstrated in Chapter 4. The action of myostatin is reported to be mediated by activin receptors (Lee and McPherron, 2001; Massagué and Chen, 2000) and the author demonstrated the mRNA expressions of activin receptors in post confluent bovine SV cells derived from adipose tissue in Chapter 4. On the other hand, the effect of activin A on the differentiation of bovine preadipocytes has been still unknown.

In this chapter, the author investigated the effect of activin A on the differentiation of bovine preadipocytes. Activin A reduced PPARγ mRNA expression and interfered with the increase of C/EBPα mRNA in bovine preadipocytes, which suppressed the induction of terminal differentiation

markers.

Follistatin is a secretory protein and it binds to activin and myostatin, which results in the prevention of their binding to the activin receptor (Lee and McPherron, 2001; Schneyer et al., 2003). The inhibitory action of myostatin on the differentiation of bovine preadipocyte was suppressed by follistatin as shown in Chapter 4. The present experiment indicated that follistatin also interfered with the suppressive action of activin A on bovine preadipocyte differentiation. Furthermore, the higher doses of follistatin stimulated the differentiation even in the presence of activin A compared with the control culture treated with neither activin A nor follistatin. The author demonstrated the mRNA expressions of activin A and myostatin in the SV cells. Therefore, endogenous activin A and/or myostatin possibly inhibited the differentiation of bovine preadipocytes.

Materials and methods

Culture of stromal-vascular cells from bovine adipose tissue

SV cells were prepared from bovine perirenal adipose tissue and cultured according to the method shown in Chapter 4. Recombinant human activin A (Wako Chemicals) and recombinant human follistatin (Genzyme Techne) was dissolved in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (Sigma) and added to the medium in the concentrations as shown in each figure during the early phase of differentiation (from day 0 to day 2) or throughout the differentiation period (from day 0 to day 8).

Glycerol-3-phosphate dehydrogenase activity

Cells were carefully washed twice with ice-cold PBS on day 8, and lysed in 25 mM Tris/ 1 mM EDTA, pH 7.5 by sonication for measurement of GPDH specific activity. GPDH activity was determined according to the method shown in Chapter 3.

Oil Red O staining

Cultures were washed twice with PBS on day 8, fixed with 10% formalin in PBS, stained with 0.5% Oil Red O and photographed.

RNA isolation

Total RNA was extracted from the SV cells according to the method shown in Chapter 4.

Northern blot analysis for the expression of adipocyte fatty acid-binding protein mRNA

The expression of aP2 mRNA in the SV cells on day 8 was analyzed by Northern blotting according to the method shown in Chapter 4.

Semi-quantitative RT-PCR for the determination of C/EBP and PPARγ mRNAs

The expression of C/EBP and PPARγ mRNAs were assessed by semi-quantitative RT-PCR according to the method shown in Chapter 4.

Detection of activin ß$_A$, myostatin, and follistatin mRNAs

The expression of activin ß$_A$, myostatin, and follistatin mRNAs in bovine adipose tissue and in SV cells on day 0 were analyzed by RT-PCR according to the method shown in Chapter 4. The PCR were conducted using Platinum PCR Super Mix (Invitrogen). The primer sets for each receptor were shown in Table 5-1. The amplification parameters consisted of denaturation at 94 °C for 30 s, annealing at the temperature (Table 5-1) for 30 s, and extension at 72 °C for 1 min, for 45 cycles. The subcloning and sequencing confirmed the PCR products to be the expected fragments of bovine activin ß$_A$, myostatin, and follistatin. The products were separated on 2% agarose gel and visualized with ethidium bromide. To avoid false-positive results from contamination by genomic DNA, samples with or without treatment of reverse transcriptase were prepared, and as a negative control, RT-PCR without RNA samples was also carried out.

Statistics

Data were expressed as means ± SE, and were statistically analyzed using student's t-test.

Table 5-1. Sequences of the primer sets

Primers	Forward	Reverse	Annealing temp, °C	Fragment size, bp	GenBank accession number
bactivin β_A	GATAGAGGACGACATCGGACG	CATCCACCACCTTCTCCGATAT	56	332	NM_174363
bmyostatin	CAAACCCATGAAAGACGGTACAA	TGAACACCCACAGCGATCTAC	63	595	AY160688
bfollistatin	TGGAAAAACCTACCGCAACG	TTCATGGCACACTCGCTAGC	60	489	L21716

Results

Differentiation of bovine preadipocytes

Oil Red O staining showed that many control cells had lipid droplets at the end of culture (Fig. 5-1-1A). However, lipid accumulation was inhibited by the treatment with 1.25 ng/ml activin A in the early phase of differentiation, and was progressively reduced at higher doses (Fig. 5-1-1A). Activin A treatment dose-dependently reduced GPDH activity (P<0.01) (Fig. 5-1-1B) and the level of aP2 mRNA (Fig. 5-1-1C). The treatment with activin A throughout the differentiation period also reduced lipid accumulation (Fig. 5-1-2A), GPDH activity (P<0.01) (Fig. 5-1-2B), and aP2 mRNA level (Fig. 5-1-2C).

Expression of C/EBP and PPARγ mRNAs

C/EBPβ mRNA expression in the control cells slightly increased after induction of differentiation, and was stable after day 2 (Fig. 5-1-3A). Activin A treatment throughout the differentiation period did not affect the expression of C/EBPβ mRNA (Fig. 5-1-3A). The control cells expressed PPARγ mRNA on day 0, then its expression moderately increased, and it reached almost maximal level on day 6 (Fig. 5-1-3B). Activin A suppressed the increase in PPARγ mRNA expression (P<0.05) during the induction of differentiation, and PPARγ mRNA level was stably low thereafter (Fig. 5-1-3B). The control cells faintly expressed C/EBPα mRNA on day 0 and its expression gradually increased after the induction of differentiation (Fig. 5-1-3C). Activin A suppressed the increase in C/EBPα mRNA level (P<0.05) (Fig. 5-1-3C).

Activin A and follistatin treatments

The author investigated whether follistatin affected the suppressive action of activin A on the differentiation of bovine preadipocytes. Activin A suppressed GPDH activity (P<0.01) when the cells were treated with activin A in the early phase of differentiation (Fig. 5-1-4). The simultaneous treatment with 50 ng/ml follistatin reversed the suppressive effect of activin A (P<0.01) (Fig. 5-1-4). Furthermore, the higher dose (300 ng/ml) of follistatin increased GPDH activity (P<0.05) compared with the control culture treated with neither activin A nor follistatin (Fig. 5-1-4). The treatment with follistatin (500 ng/ml) alone increased GPDH activity (P=0.055) compared with the control culture (Fig. 5-1-4).

Expressions of activin β_A, myostatin, and follistatin mRNAs

Expressions of bovine activin β_A, myostatin, and follistatin mRNAs were examined by RT-PCR in bovine adipose tissue and in postconfluent bovine SV cells. We detected all these mRNA expressions in both adipose tissue and in the SV cells (Fig. 5-1-5). No bands were detected in the absence of reverse transcriptase or RNA samples during the synthesis of cDNA (data not shown)
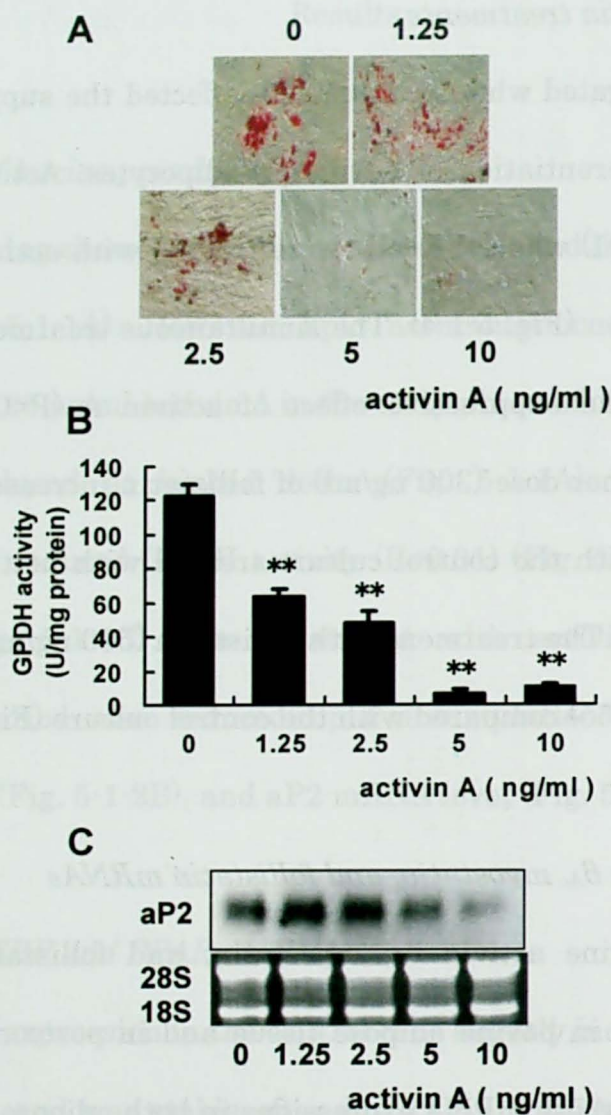
Fig. 5-1-1. Effect of activin A treatment in the early phase on bovine preadipocyte differentiation.

Bovine SV cells were treated with activin A for 2 days (from day 0 to day 2) and cells were harvested at the end of differentiation period (day 8). Intracellular lipid was stained with Oil Red O (A). GPDH activity per mg protein was determined (B). The expression of aP2 mRNA was measured by Northern blot analysis. Ethidium bromide staining demonstrates the RNA loading (C). Values are means ± SE for four replicated cultures. **P<0.01 vs culture treated without activin A.
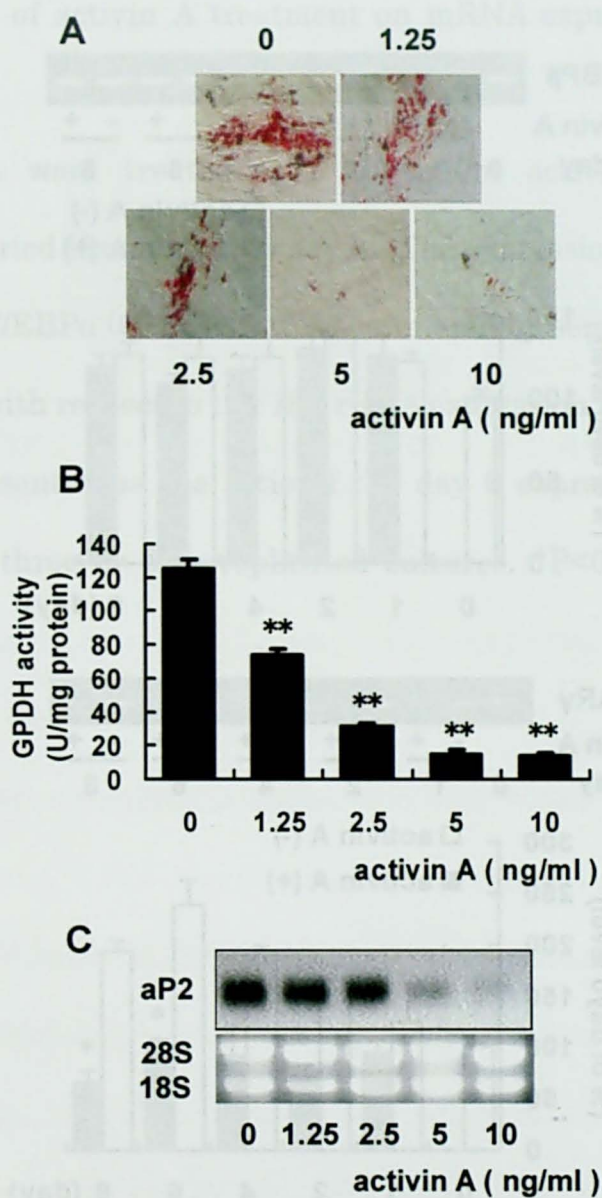
Fig. 5-1-2. Effect of activin A treatment throughout the differentiation period on bovine preadipocyte differentiation.

Bovine SV cells were treated with activin A for 8 days (from day 0 to day 8). Intracellular lipid (A), GPDH activity (B), and Northern blot analysis for the expressions of aP2 mRNA (C) were determined as described in the Fig.5-1-1. legend. Values are means ± SE for four replicated cultures. **P<0.01 vs culture treated without activin A.

A

C/EBPβ

activin A   - +   - +   - +   - +   - +

day   0   1   2   4   6   8

□ activin A (-)
■ activin A (+)

B

PPARγ

activin A   - +   - +   - +   - +   - +

day   0   1   2   4   6   8

□ activin A (-)
■ activin A (+)

C

C/EBPα

activin A   - +   - +   - +   - +   - +

day   0   1   2   4   6   8
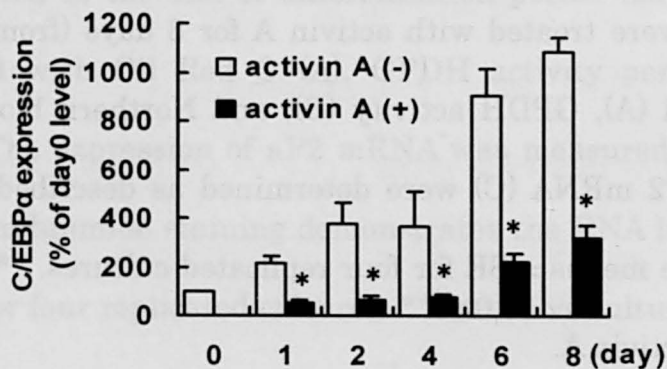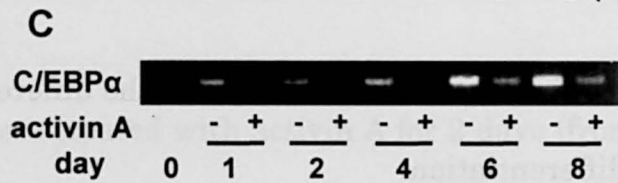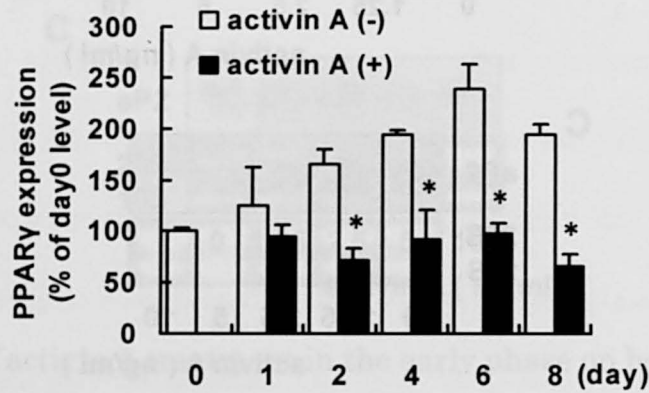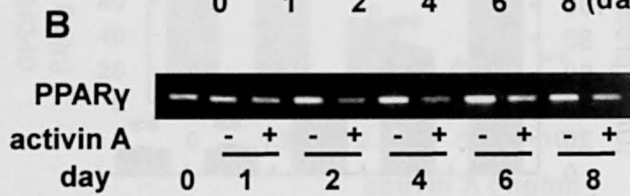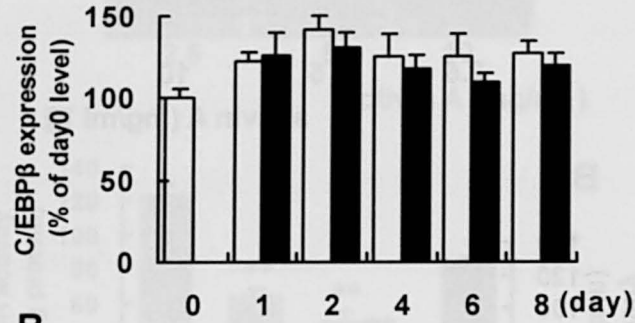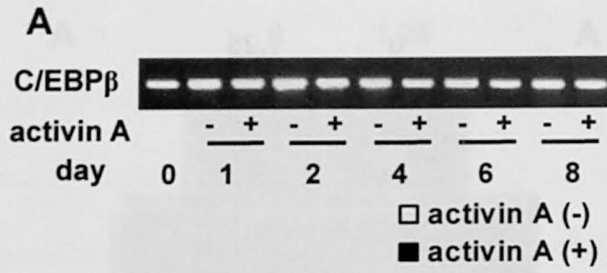
□ activin A (-)
■ activin A (+)

Fig. 5-1-3. Effect of activin A treatment on mRNA expressions of C/EBP and PPARγ.

Bovine SV cells were treated with 10 ng/ml activin A throughout the differentiation period (from day 0 to day 8). The expression levels of C/EBPβ (A), PPARγ (B), and C/EBPα (C) mRNA were estimated by semi-quantitative RT-PCR and normalized with respect to the 18S rRNA expression level. The relative gene expression is presented as the ratio of the day 0 expression level. Values are means ± SE for three or four replicated cultures. *P<0.05 vs culture treated without activin A.
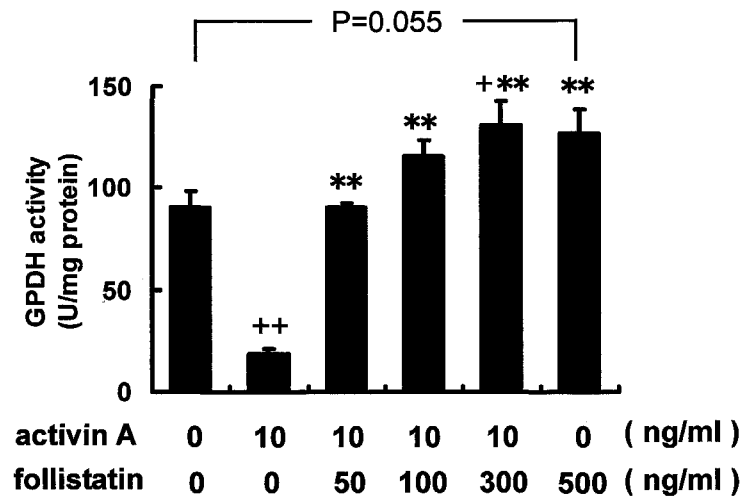
Fig. 5-1-4. Effect of activin A and follistatin treatments on bovine preadipocyte differentiation.

Bovine SV cells were treated with 10 ng/ml activin A with follistatin in various concentrations or 500 ng/ml follistatin alone in the early phase (from day 0 to day 2) and cells were harvested at the end of culture (day 8). GPDH activity was determined as described in the Fig. 1 legend. Values are means ± SE for four replicated cultures. $^{+}P<0.05$, $^{++}P<0.01$ vs culture treated neither activin A nor follistatin; $^{**}P<0.01$ vs culture treated with activin A alone.

activin β_A   myostatin   follistatin

tissue   SV   tissue   SV   tissue   SV
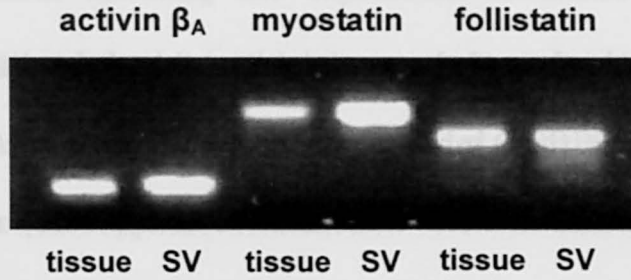
Fig. 5-1-5. Expression of activin β_A, myostatin, and follistatin mRNAs in bovine

adipose tissue and in stromal-vascular (SV) cells.

Total RNA was extracted from bovine adipose tissue and post confluent (day 0)

bovine SV cells, and RT-PCR was performed using primer sets for bactivinβ_A,

bmyostatin, and bfollistatin. The products were separated on 2% agarose gel and

visualized with ethidium bromide.

The present study showed that activin A inhibited the differentiation of bovine preadipocytes and this inhibitory effect of activin A was reversed by simultaneous treatment with follistatin. Furthermore, the higher doses of follistatin stimulated the differentiation even in the presence of activin A compared with the control culture treated with neither activin A nor follistatin. The SV cells in the present study expressed activin A and myostatin mRNAs. These results suggest that follistatin also interferes with endogenous activin A and/or myostatin that inhibit the differentiation of preadipocytes in the SV cells derived from bovine adipose tissue.

The bovine control cells almost stably expressed C/EBPβ mRNA, the expression of PPARγ was relatively high on day 0 and moderately increased thereafter, and C/EBPα mRNA level was faint on day 0 and drastically increased between day 4 and day 6.adipose tissue.

The present experiment showed that C/EBPβ mRNA in the bovine control cells was stably expressed until day 6, the expression of PPARγ was moderately increased after induction of differentiation, and C/EBPα mRNA level was drastically increased between day 4 and day 6. These results were almost similar to the results in Chapter 4.

In the present study, activin A treatment in the early phase of differentiation reduced lipid accumulation, GPDH activity, and aP2 mRNA level, and its treatment throughout the differentiation period also reduced these terminal differentiation markers. Activin A treatment throughout the differentiation

period suppressed the increase in PPARɣ mRNA expression and the induction of C/EBPα mRNA without affecting the expression of C/EBPβ mRNA, which suggests that activin A suppressed bovine preadipocyte differentiation via inhibiting the transcriptional cascade downstream of C/EBPβ mRNA expression. Activin A was reported to suppress the function of C/EBPβ in human hepatocyte culture, which was mediated by Smad3 protein that interacted with the DNA binding domain of C/EBPβ and repressed the transactivation function of C/EBPβ (Zauberman et al., 2001). TGF-β also inhibited the function of C/EBPβ mediated by Smad3 protein, which decreased gene expression of PPARɣ, and thus TGF-β inhibited adipogenesis (Choy and Derynck, 2003). Therefore, activin A possibly prevents bovine preadipocyte differentiation via Smad3-mediated impairment of the transactivation function of C/EBPβ.

During human pregnancy, serum concentration of activin A is reported to increase progressively (Petraglia et al., 1995). On the other hand, activin A is normally present in relatively low amounts in the circulation (below 0.1 ng/ml) in adult sheep (Jenkin et al., 2001; Jones et al., 2004), and the activin A level did not change throughout the gestation (McFarlane O'Shea et al., 1998). Vejda et al. (2002) reported that activin βA was highly expressed in adipose tissue in rat, and the author also found the mRNA expression of activin βA in adipose tissue in cattle, which predicting that activin A affects adipogenesis through autocrine/paracrine manner. Serum concentration of activin A in cattle has not been studied, however, locally produced activin A may be highly involved in the preadipocyte differentiation than serum activin A in ruminants.

The present experiment indicated that follistatin reversed the inhibitory effect

of activin A on bovine preadipocyte differentiation. Follistatin is known to capture activin A, which neutralizes its activity (Schneyer et al., 2003). Follistatin is likely to attenuate activin A action on preadipocyte differentiation through forming the inactive complex.

In the present study, the high dose (300 ng/ml) of follistatin increased GPDH activity in the presence of activin A compared with the control culture treated with neither activin A nor follistatin. Furthermore, the treatment with 500 ng/ml follistatin alone stimulated the differentiation compared with the control culture. The present study indicated that the bovine SV cells expressed both activin β_A and myostatin mRNAs. Follistatin also captures myostatin and inhibits its action (Lee and McPherron, 2001). Endogenous activin A and/or myostatin probably inhibited preadipocyte differentiation in the SV cell culture, and thus exogenous follistatin promoted adipogenesis. The expression of follistatin, as well as activin A, is widely distributed in gonadal and non-gonadal tissues including adipose tissue (Schneider et al., 2000). The author demonstrated the expression of follistatin in bovine adipose tissue. Myostatin mRNA is found primarily in skeletal muscle (McPherron et al., 1997), however, it was also detected in bovine adipose tissue. Furthermore, the author reported in Chapter 4 that myostatin inhibited the differentiation of bovine preadipocytes, and this inhibitory effect of myostatin was interfered with the simultaneous treatment of follistatin. The interaction between activin A and follistatin, or myostatin and follistatin may exist in the adipose tissue. The physiological balance of these autocrine/paracrine factors possibly regulates preadipocyte differentiation in adipose tissue.

In summary, the results presented in this section showed that activin A

inhibited bovine preadipocyte differentiation, and this inhibitory effect of activin A was mediated by the reduction of PPARγ and C/EBPα mRNA levels. Additionally, follistatin affected bovine preadipocyte differentiation, which suggests that follistatin modulates the action of activin A and/or myostatin on preadipocyte differentiation.

The mechanism of the inhibitory effect of activin A

on the differentiation of 3T3-L1preadipocyte

Introduction

Activin A, a member of the transforming growth factor-β (TGF-β) superfamily, is homodimer of activin β_A subunit. Recently, the expressions of activin β_A (Meunier et al., 1988; Tuuri et al., 1994) and activin receptor mRNAs (Mathews, 1994) were found in many tissues throughout the body. Especially, activin β_A was highly expressed in adipose tissue (Vejda et al., 2002).

3T3-L1 preadipocyte is one of the most well-characterized and reliable models for studying adipogenesis. Upon reaching confluence, the treatment with adipogenic agents induces mitotic clonal expansion (MacDougald and Lane, 1995; Richon et al., 1997). CCAAT/enhancer binding protein (C/EBP) β is highly expressed after the treatment with adipogenic agents (Lane et al., 1999) and C/EBPβ activates the expression of peroxisome proliferator-activated receptor (PPAR) γ and C/EBPα mRNAs that coordinately activate the transcription of adipocyte-specific genes (Rosen et al., 2000).

In Section 1 in Chapter 5, the author demonstrated that activin A inhibited the differentiation of bovine preadipocyte. Additionally, the author reported that myostatin, a member of TGF-β superfamily also inhibited the differentiation of bovine preadipocyte in Chapter 4. The inhibitory effects of activin A and

myostatin on the differentiation of bovine preadipocyte were both mediated by the suppression of PPAR γ and C/EBPα mRNA expression. However, further mechanism that activin A and myostatin inhibit preadipocyte differentiation has been still unclear.

Zauberman et al. (2001) reported that activin A inhibited haptoglobin expression via suppressing C/EBPß function in human hepatoma cells. Thus, activin A may inhibit adipocyte differentiation via suppressing C/EBPß function.

In this chapter, the author investigated the mechanism of the inhibitory effect of activin A on the differentiation of 3T3-L1 preadipocyte. Activin A inhibited the early phase of differentiation via suppressing PPARγ and C/EBPα mRNA expression. Activin A interfered with the DNA binding activity of C/EBPß, which was considered to suppress the differentiation.

Materials and methods

Cell culture

3T3-L1 preadipocyte were cultured according to the method shown in Chapter 3. Recombinant human activin A (Wako Chemicals) was dissolved in PBS with 0.1% bovine serum albumin (Sigma) and added to the medium in various concentrations as shown in each figure throughout the differentiation period (from day 0 to day 8), during the early phase of differentiation (from day 0 to day 2) or during the late phase of differentiation (from day 2 to day 8).

Quantification of mitotic clonal expansion

Cells were treated with activin A during the early phase of differentiation, trypsinized in trypsin / EDTA and resuspended in DMEM. Then the number of cells was counted on Thoma-type hemocytometer.

Glycerol-3-phosphate dehydrogenase activity

Cells were carefully washed twice with ice-cold PBS on day 8, and lysed in 25 mM Tris/1 mM EDTA, pH 7.5 for measurement of GPDH specific activity. GPDH activity was determined according to the method shown in Chapter 3.

Oil Red O staining

Cultures were washed twice with PBS on day 8, fixed with 10% formalin in PBS, stained with 0.5% Oil Red O and photographed.

RNA isolation

Total RNA was extracted from 3T3-L1 cells according to the method shown in Chapter 4.

Detection of activin receptor mRNAs in 3T3-L1 preadipocyte

The expression of activin receptor mRNAs encoding type I (ActRI and ActRIB) and type II (ActRII and ActRIIB) receptors on day 0 were examined by RT-PCR. Single-strand cDNA was synthesized using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacture's protocol. The PCR were conducted using Platinum PCR Super Mix (Invitrogen). The primer

sets for each receptor were shown in Table 5-2. Thirty-five cycles were selected for detection. The products were separated on 2% agarose gel and visualized with ethidium bromide.

Northern blot analysis for the expression of aP2 mRNA

RNA sample (4 µg) was separated in 1% agarose gels containing 6.7% formaldehyde and transferred to a Hybond N+ membrane (Amersham Biosciences, NJ, USA). cDNA fragment of mouse adipocyte fatty acid-binding protein (aP2) gene was amplified using specific primers (Table 5-2.) and cDNA probe was labeled with digoxigenin-dUTP by using random priming (Roche Diagnostics). The band corresponding to aP2 mRNA was detected using DIG Luminescant Detection Kit for Nucleic Acids (Roche Diagnostics).

Semi-quantitative RT-PCR for the determination of C/EBP and PPARγ mRNAs

The expression of C/EBP and PPARγ mRNAs were assessed by semi-quantitative RT-PCR. The thermal amplifications of C/EBP, PPARγ, and 18S rRNA fragments were conducted using Platinum PCR Super Mix (Invitrogen). The primer sets were shown in Table 5-2. PCR conditions were optimized for detection within a linear range. Twenty-eight cycles were selected for experimental detection of C/EBPβ, 26 cycles for PPARγ, 30 cycles for C/EBPα, and 24 cycles for 18S rRNA. The level of 18S rRNA was adopted as the internal standards for the determination of targeted mRNA levels.

Western immunoblot analysis

3T3-L1 cells were harvested and lysed in 25 mM Tris / 1 mM EDTA, pH 7.5 by sonication. Protein concentration of cell lysate was determined and 20 µg of protein was subjected to 12.5% SDS-PAGE and transferred to Immobilon-P membrane (Millipore). After blocking with 3% skim milk in PBS, membrane was incubated with primary antibody, polyclonal anti- C/EBPß (Santa Cruz Biotechnology, CA, USA; 1/500) for 1 h, followed by incubation with secondary antibody conjugated to horseradish peroxidase, anti-rabbit IgG (Amersham Biosciences, 1/2000) for 30 min. Visualization of the second antibody binding was performed by chemiluminescence with the ECL plus Western blotting detection system (Amersham Biosciences).

Electrophoretic mobility shift assay (EMSA)

3T3-L1 cells treated with 20 ng/ml activin A in 60-mm dishes (Corning) were carefully rinsed twice with ice-cold 10 mM HEPES buffer, pH 7.5 on day 2. Then the cells were incubated with 10 mM HEPES buffer for 15 min on ice and scraped. The harvested cells were lysed by the dounce homogenizer with 15 strokes. After centrifugation at 10,000 rpm for 3 min at 4 °C, the supernatant was discarded. After a crude nuclear pellet was washed twice with ice-cold 25 mM HEPES / 3 mMgCl$_2$ / 1 mM DTT, pH 7.5., the pellet was resuspended in ice-cold 25 mM HEPES / 0.4 mM KCl / 1mM DTT, pH 7.5. Then the pellet was incubated on ice for 30 min with occasional vortexing. After the incubation, glycerol was added to become 10% of final concentration and the reaction buffer containing nuclear extract was centrifuged at 12,000 rpm for 1 h at 4 °C. The supernatant was

stored as nuclear extract at -80 °C. Protein concentration was measured with the method of Lowry et al. (1951). Five μg of nuclear extract protein was subjected to electrophoretic mobility shift assay (EMSA) using EMSA kit for C/EBP (Panomics, CA, USA), according to the manufacture's protocol. The sequence of C/EBP probe was 5'-TGCAGATTGCGCAATCTGCA-3', which contained the binding site of C/EBPβ. Polyclonal anti- C/EBPβ (Santa Cruz Biotechnology) was used for the determination of the complex of C/EBPβ and C/EBP probe.

Statistics

Data were expressed as means ± SE, and were statistically analyzed using student's t-test. Statistical significance was set at P<0.05.

Table 5-2. Sequences of the primer sets

Primers	Forward	Reverse	Annealing temp, °C	Fragment size, bp	GenBank accession number
mActRI	ATGACTACCTTCAGCTCACT	CTCGCCAGAGAAGTTAATG	60	542	BC058718
mActRIB	ACCGCTACACAGTGACCATT	TCTTCACATCTTCCTGCACG	60	630	NM_007395
mActRII	CTTAAGGCTAATGTGGTCTC	GACTAGATTCTTTGGGAGGA	58	703	M65287
mActRIIB	ATCGTCATCGGAAACCTCCC	CAGCCAGTGATCCTTAATC	59	929	M84120
mC/EBPα	AGTCGGCCGACTTCTACGAG	GATGTAGGCGCTGATGT	62	200	M62362
mC/EBPβ	GCAAGAGCCGCGACAAG	GGCTCGGGCAGCCTGCTT	56	154	X62600
mPPARγ	GGAGATCTCCAGTGATATCGACCA	ACGGCTTCTACGGATCGAAACT	56	294	U01664
maP2	TCTCACCTGGAAGACAGCTCCTCCTCG	TTGGTAGGTCCGGAGAAGGAAACCGAG	56	463	K02109
m18S rRNA	GGGAGCCTGAGAAACGGC	CCGCTCCCAAGATCCAACTAC	56	262	X00686

Expression of activin receptor mRNAs

Expression of mRNAs coding type I activin receptor (ActRI and ActRIB) and type II activin receptor (ActRII and ActRIIB) were examined by RT-PCR. These receptor mRNA expressions were detected in the postconfluent cells (Fig. 5-2-1).

Mitotic clonal expansion

The effect of activin A on the mitotic clonal expansion was examined (Fig. 5-2-2). The number of preadipocytes increased almost 2-fold when differentiation was induced without activin A, which was in accordance with the previous reports (Lyle et al., 1998; Liu et al., 2002). Activin A did not affect the increase in cell number after induction of differentiation.

Differentiation of preadipocyte

The effects of activin A on the induction of terminal differentiation markers at the end of differentiation period were investigated. Oil Red O staining showed that control cells had many lipid droplets. However, lipid accumulation was inhibited by the treatment with 10 ng/ml activin A throughout the differentiation period, and lipid accumulation was progressively reduced at higher doses (Fig. 5-2-3A). Activin A treatment dose-dependently reduced GPDH activity (Fig. 5-2-3B) and the level of aP2 mRNA (Fig. 5-2-3C). Activin A treatment during the early phase of differentiation also reduced GPDH activity (Fig. 5-2-4A) and aP2 mRNA level (Fig. 5-2-4B), although the reduction of aP2 mRNA level was small

compared with the treatment throughout the differentiation period. Activin A treatment during the late phase of differentiation modestly decreased GPDH activity (Fig. 5-2-5A) and aP2 mRNA level (Fig. 5-2-5B).

Expressions of C/EBP and PPARγ mRNA

The expression of C/EBPβ mRNA drastically increased 12 h after the induction of differentiation in the control cells, reached maximal level after 24 h, and then gradually declined (Fig. 5-2-6A). Activin A treatment did not affect the expression of C/EBPβ mRNA. The expression of PPARγ mRNA gradually increased from 12 h after the induction of differentiation and reached maximal level after 6 days in the control cells but the treatment with activin A suppressed the expression of PPARγ mRNA (Fig. 5-2-6B). C/EBPα mRNA expression gradually increased from 24 h after the induction of differentiation in the control cells but the treatment with activin A suppressed C/EBPα mRNA expression (Fig. 5-2-6C). However, the expression of PPARγ and C/EBPα mRNAs partly recovered after activin A was withdrawn.

Expression of C/EBPβ protein

The protein expression of C/EBPβ was also examined by Western immunoblot analysis. There are two active isoforms of C/EBPβ protein of which molecular weights were 35 and 38 kDa. These isoforms increased within 12 h after the induction of differentiation, and then gradually declined in the control cells (Fig. 5-2-7). Activin A treatment did not affect the expression of C/EBPβ protein, which was in accordance with its mRNA level.
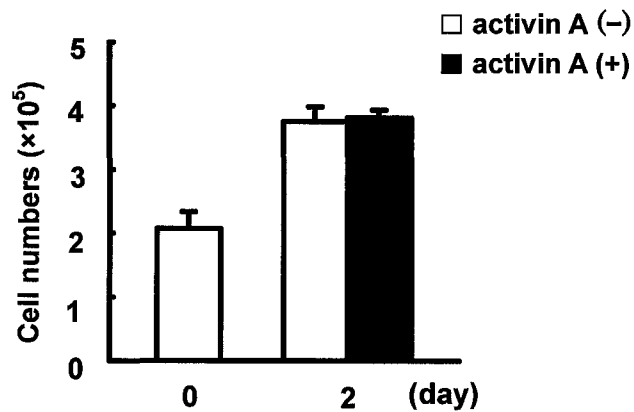
DNA binding activity of C/EBPβ

Binding activity of C/EBPβ to C/EBP probe was examined by EMSA. When C/EBP probe was incubated with nuclear extract prepared from control cells treated without activin A, a clear band was detected, however, the chemiluminescence intensity of this band was diminished by the addition of C/EBPβ antibody, which confirmed the band was a complex of C/EBPβ and C/EBP probe (Fig. 5-2-8). When the cells were treated with activin A during the induction of differentiation (from day 0 to day 2), the formation of this complex was markedly reduced compared with the control cells (Fig 5-2-8).

Fig. 5-2-1. Expression of activin receptor mRNA in 3T3-L1 preadipocyte.

Post confluent (day 0) 3T3-L1 preadipocytes were harvested and RT-PCR was performed using primer sets for ActRI (I), ActRIB (IB), ActRII (II), or ActRIIB (IIB). The products were separated on 2% agarose gel and visualized with ethidium bromide.

Fig. 5-2-2. Effect of activin A treatment on mitotic clonal expansion during 3T3-L1 preadipocyte differentiation induction process.

3T3-L1 cells treated with 20 ng/ml activin A during differentiation induction phase (from day 0 to day 2) were trypsinized in Trypsin / EDTA, resuspended in DMEM. Cell numbers were counted on Thoma-type hemocytometer. Values are means ± SE for triplicated cultures.
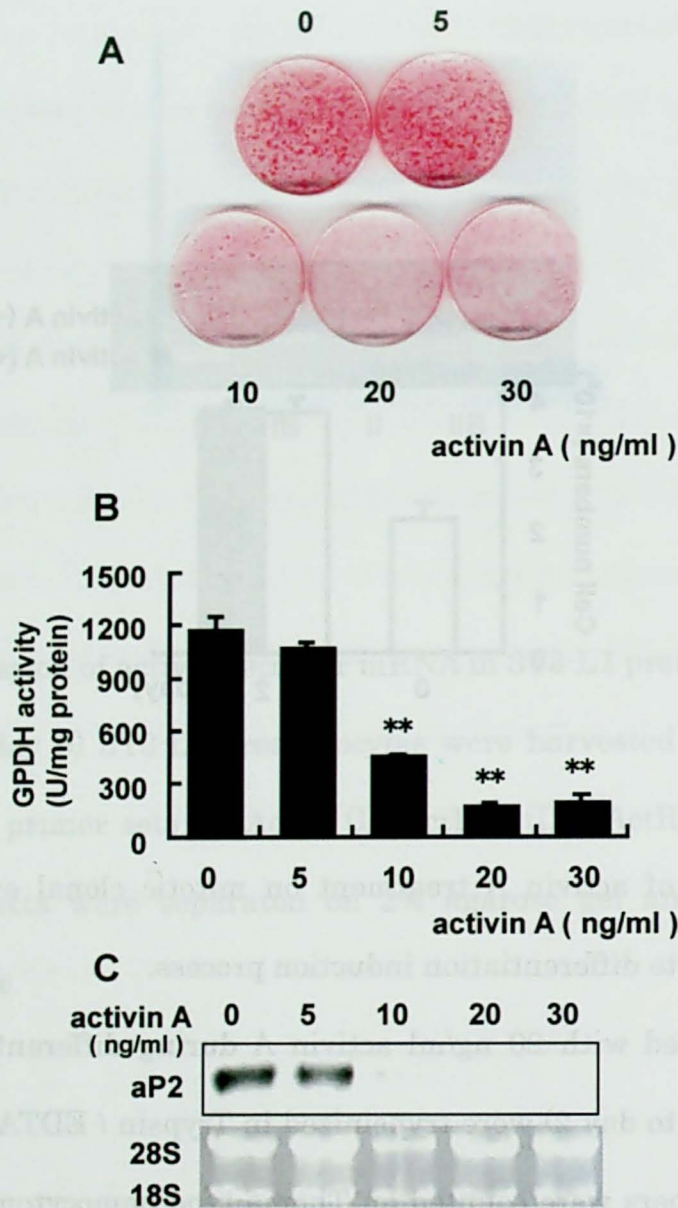
Fig. 5-2-3. Effect of activin A treatment throughout the differentiation period on 3T3-L1 preadipocyte differentiation.

The differentiating 3T3-L1 cells were treated with activin A in various concentrations for 8 days (from day 0 to day 8). Intracellular lipid was stained with Oil Red O (A). GPDH activity per mg protein was determined (B). The expression of aP2 mRNA was measured by Northern blot analysis. Ethidium bromide staining demonstrates the RNA loading (C). Values are means ± SE for triplicated cultures. **P<0.01 vs culture treated without activin A.

A

B
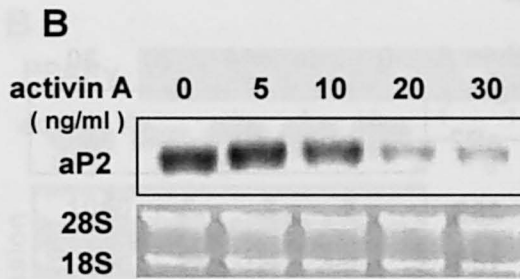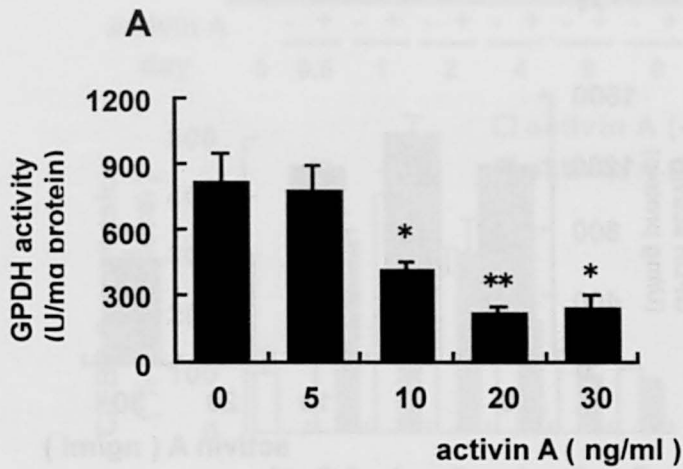
Fig. 5-2-4. Effect of activin A treatment in the early phase on 3T3-L1 preadipocyte differentiation.

The differentiating 3T3-L1 cells were treated with activin A in various concentrations for 2 days (from day 0 to day 2) and cells were harvested at the end of differentiation period (day 8). GPDH activity (A) and Northern blot analysis for the expression of aP2 mRNA (B) were determined as describ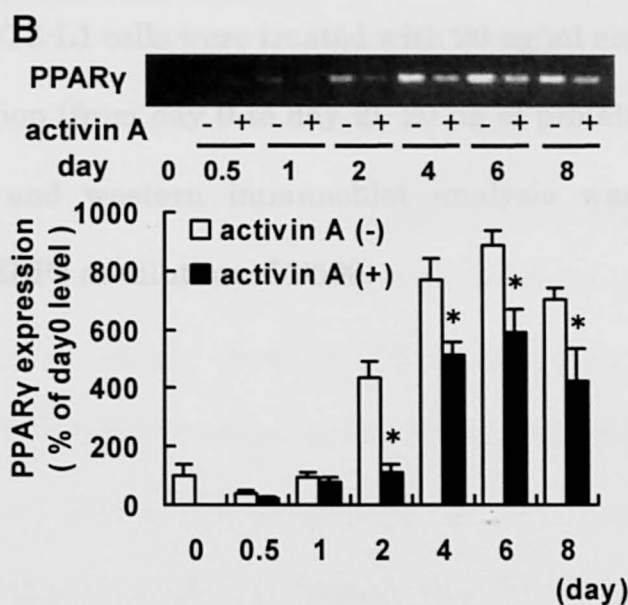ed in the Fig. 5-2-3 legend. Values are means ± SE for triplicated cultures. *P<0.05, **P<0.01 vs culture treated without activin A.
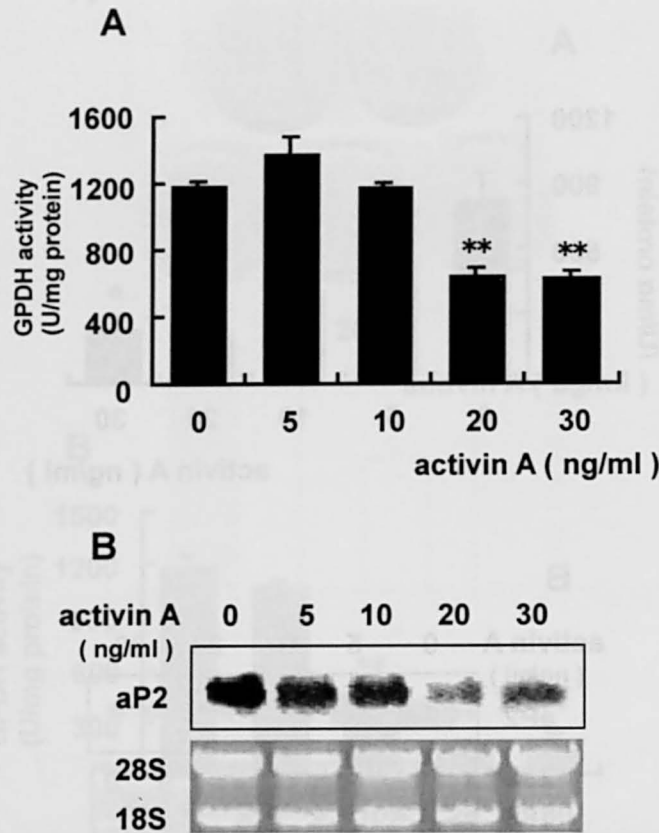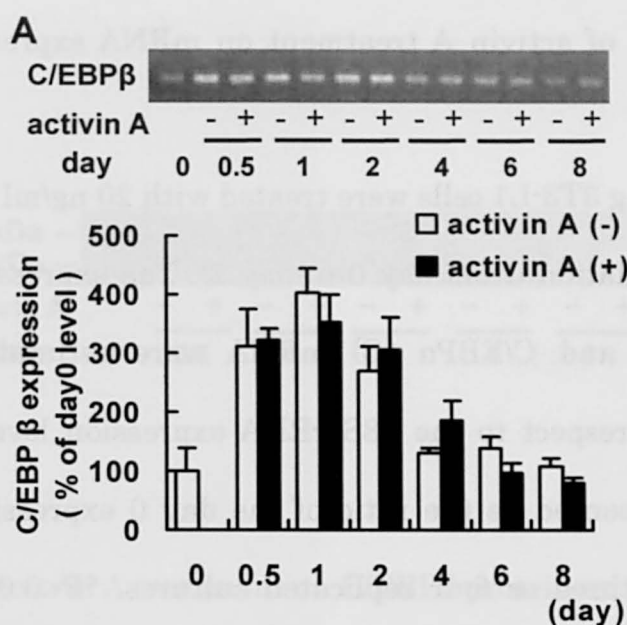
Fig. 5-2-5. Effect of activin A treatment during the late phase on 3T3-L1 preadipocyte differentiation.

The differentiating 3T3-L1 cells were treated with activin A in various concentrations in the late phase (from day 2 to day 8). GPDH activity (A) and Northern blot analysis for the expression of aP2 mRNA (B) were determined as described in the Fig. 5-2-3 legend. Values are means ± SE for triplicated cultures. **P<0.01 vs culture treated without activin A.
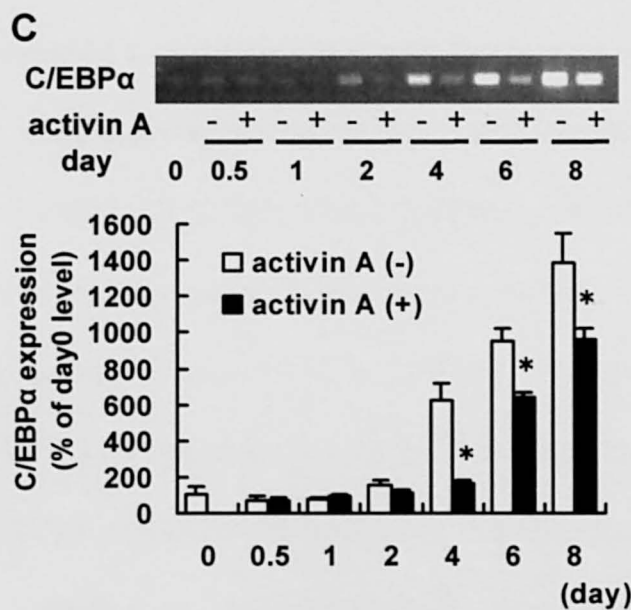
Fig. 5-2-6. Effect of activin A treatment on mRNA expression of C/EBPs and PPARγ.

The differentiating 3T3-L1 cells were treated with 20 ng/ml activin A in the early phase of differentiation (from day 0 to day 2). The expression levels of C/EBPβ (A), PPARγ (B), and C/EBPα (C) mRNA were estimated by RT-PCR and normalized with respect to the 18S rRNA expression level. The relative gene expression is presented as the ratio of the day 0 expression level. Values are means ± SE for three or four replicated cultures. *$P<0.05$ vs culture treated without activin A.

38kDa —
35kDa —
activin A   – + – + – + – + – +
day   0   0.5   1   2   4   6
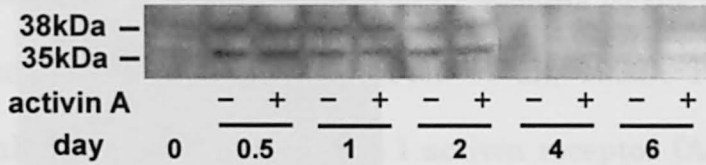
Fig. 5-2-7. Effect of activin A treatment on C/EBPß protein expression.

The differentiating 3T3-L1 cells were treated with 20 ng/ml activin A in the early phase of differentiation (from day 0 to day 2). 20 µg of protein was subjected to 12.5% SDS-PAGE, and western immunoblot analysis was performed with antibody against C/EBPß at dilution of 1:500.
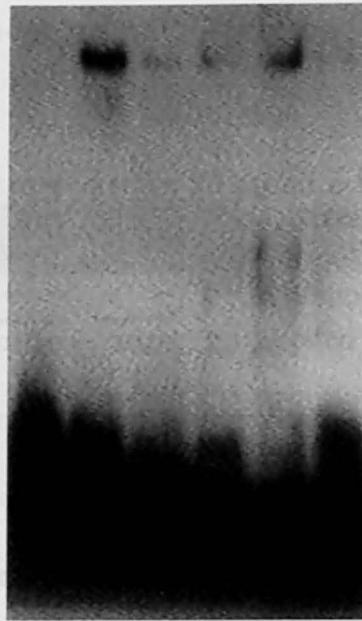
nuclear extract (0 ng/ml activin A)	–	+	–	+	+	–
nuclear extract (20 ng/ml activin A)	–	–	+	–	–	+
labeled probe	+	+	+	+	+	+
unlabeled probe	–	–	–	+	–	–
anti-C/EBPβ antibody	–	–	–	–	+	+

Fig. 5-2-8. Effect of activin A treatment on the C/EBPß binding activity to C/EBP response element.

The differentiating 3T3-L1 cells were treated with 20 ng/ml activin A in the early phase of differentiation (from day 0 to day 2) and harvested on day 2. 5 µg of nuclear extract protein was subjected to electrophoretic mobility shift assay (EMSA).

Discussion

Activin A initiates its biological functions by binding to the cell surface type I and type II receptors (Mathews, 1994; Piek et al., 1999). The present study demonstrated mRNA expressions of type I activin receptor (ActRI and ActRIB) and type II activin receptor (ActRII and ActRIIB) in post confluent 3T3-L1 preadipocyte. These results suggest that 3T3-L1 preadipocyte can respond to activin A.

GPDH activity and aP2 mRNA level were decreased in the cultures treated with activin A in the early differentiation period, the step of commitment to differentiation in 3T3-L1 preadipocyte (Gregoire et al., 1998). C/EBPs and PPARγ are key transcription factors in the signaling cascade during adipocyte differentiation (Gregoire et al., 1998). C/EBPβ is induced by 3-isobutyl-1-methylxanthine in the adipogenic agents (Yeh et al., 1995) and its expression reaches maximal level within 4 h (Lane et al., 1999). Tang and Lane (1999) reported that C/EBPβ acquired DNA binding activity after 10-12 h lag period, and C/EBPβ participated in the regulation of mitotic clonal expansion, which was considered as a critical event for adipogenesis. Thereafter, C/EBPβ transcriptionally activates both PPARγ and C/EBPα expressions (Gregoire et al., 1998; Lane et al., 1999). PPARγ and C/EBPα coordinately activate the expression of adipocyte-specific genes (Gregoire et al., 1998). The present experiment indicated that activin A treatment in the early differentiation period inhibited the expression of PPARγ and C/EBPα mRNAs but did not affect the expression of C/EBPβ mRNA and its protein level. In Section 1 in Chapter 5, the author

showed that activin A inhibited bovine preadipocyte differentiation via affecting downstream of C/EBPß mRNA expression and suggested that activin A possibly prevented bovine preadipocyte differentiation via Smad3-mediated impairment of the transactivation function of C/EBPß. In the present study, the effect of activin A on the 3T3-L1 preadipocyte differentiation was not affected by the different expression patterns of the critical transcription factors between 3T3-L1 cells and bovine SV cells, that was similar to the effect of myostatin on the differentiation of 3T3-L1 preadipocytes (Kim et al., 2001) and bovine preadipocytes in the study in Chapter 3.

In the present study, activin A treatment in the early phase of differentiation reduced the formation of C/EBPß and C/EBP probe complex, which suggested that activin A inhibited adipogenesis via affecting the DNA binding ability of C/EBPß. TGF-ß inhibited the function of C/EBPß, which decreased gene expression of PPARγ and thus TGF-ß inhibited adipogenesis (Choy and Derynck, 2003). The inhibitory actions of TGF-ß were mediated by Smad3 protein, a signal effecter of TGF-ß, that interacted with the DNA binding domain of C/EBPß and repressed the transactivation function of C/EBPß, although the DNA binding ability of C/EBPß was not affected by Smad3 (Choy and Derynck, 2003). On the other hand, activin A was reported to suppress haptoglobin promoter activation mediated by C/EBPß in human hepatoma cells, which was involved in the repression of DNA binding activity of C/EBPß by the activation of Smad3 protein (Zauberman et al., 2001). Therefore, in the present study, activin A possibly prevented adipogenesis in the early phase via Smad3-mediated impairment of the DNA binding ability of C/EBPß, and thus of the transactivation function of

C/EBPβ.

Activin A did not affect mitotic clonal expansion in the present experiment. Ignotz and Massagué (1985) showed that TGF-β did not affect mitotic clonal expansion but inhibited adipogenesis when the cells were exposed to TGF-β in the early phase of differentiation. Thus, the activation of Smad3 by activin A or TGF-β possibly suppresses adipocyte differentiation without affecting mitotic clonal expansion. C/EBPβ was reported to localize to centromere through consensus C/EBP-binding sites in centromeric satellite DNA at the onset of mitotic clonal expansion (Tang and Lane, 1999) though the mechanism of C/EBPβ action on mitotic clonal expansion has not been clear. The mechanism of C/EBPβ action on mitotic clonal expansion might be different from that on the expression of PPARγ and C/EBPα mRNAs. Otherwise, as mentioned later, activin A might suppress the expression of PPARγ and C/EBPα mRNAs and adipogenesis via other mechanisms than the impairment of C/EBPβ function.

The reduction of GPDH activity and aP2 mRNA level was also observed in the cultures treated with activin A during the late phase though the treatment during the late phase was less effective than the treatment during the other phases. Moreover, the expression of PPARγ and C/EBPα mRNAs partly recovered after activin A was withdrawn in the late differentiation period and the expression of aP2 mRNA was lower in the culture treated with activin A throughout the differentiation period than in the culture treated during the early differentiation period. Additionally, the suppressive effects on the expression of PPARγ and C/EBPα mRNAs were also observed in the cells treated during the late differentiation period (data not shown). Thus, activin A probably inhibits

adipogenesis via suppressing the expression of PPARɣ and C/EBPα mRNAs even in the late period. The level of C/EBPβ is very low in the late phase of differentiation, which suggests that activin A interferes with the expression of PPARɣ and C/EBPα mRNAs through other mechanisms than the impairment of C/EBPβ function in the late period. Therefore, it can postulate that activin A also suppresses the expression of these transcription factors in the early differentiation period via the same mechanism observed in the late differentiation period. However, there have not been any reports showing that expression of PPARɣ and C/EBPα mRNAs are mediated by other factors than C/EBPβ in the early phase of differentiation. Further study is necessary for clarifying this other mechanisms of activin A action on the adipogenesis.

Vejda et al. (2002) reported that activin A was highly expressed in adipose tissue, predicting that activin A affects adipogenesis in adipose tissue through autocrine/paracrine manner. On the other hand, serum concentration of activin A ranged between 4.8 ng/ml and 25.4 ng/ml in women with normal pregnant (Petraglia et al., 1995). These concentrations corresponded with doses that affected adipogenesis in the present experiment. Therefore, serum activin A may affect adipogenesis as an endocrine factor.

In summary, the results presented here showed that activin A inhibited adipogenesis and this inhibitory effect was mediated by the suppression of the DNA binding ability of C/EBPβ, and thus by the reduction of PPARɣ and C/EBPα mRNA levels.

Summary

Beef marbling is characterized by adipose tissue deposition within skeletal muscle in cattle. Japanese black cattle (Wagyu) is well known for its ability to produce high marbling, and fat deposition in the muscle is not necessarily reflected by systemic fat metabolism in this breed. As intramuscular preadipocytes are surrounded by mature skeletal muscle myofibers, it is predicted that paracrine factors secreted from muscle fibers may regulate the differentiation of preadipocytes via intercellular interactions.

In the present study, the conditioned medium from C_2C_{12} myocytes mostly suppressed the 3T3-L1 preadipocyte differentiation in the early phase that is the step of commitment to differentiate (Chapter 3). During the induction of differentiation, C/EBPβ is expressed immediately (within 2-4 hours), and C/EBPβ transcriptionally activats PPARγ and C/EBPα expression Thereafter, PPARγ and C/EBPα upregulate each other to maintain their expression despite the reduction of C/EBPβ level. PPARγ and C/EBPα coordinately activate the expression of adipocyte-specific genes. Myostatin, which is essential for proper regulation of skeletal muscle mass, is known to be expressed mainly in skeletal muscle. Furthermore, myostatin is reported to be expressed in C_2C_{12} myotubes and to be secreted into the culture medium. Additionally, myostatin is reported to inhibit the differentiation of 3T3-L1 preadipocytes via affecting the expression of PPARγ and C/EBPα. Based on these reports and results in Chapter 3, myostatin was thought to be a candidate for the paracrine factor from C_2C_{12} myotubes that inhibited the differentiation of 3T3-L1 preadipocytes. To examine this possibility,

the differentiating 3T3-L1 cells were co-treated with the conditioned medium and follistatin that binds to myostatin and activin and inhibit their action. Follistatin reversed the inhibitory effect of the conditioned medium on the 3T3-L1 preadipocyte differentiation in a dose dependent manner, which suggested that myostatin and/or activin in conditioned medium possibly inhibited the preadipocyte differentiation (Chapter 3).

To investigate the effect of myostatin on the differentiation of bovine preadipocytes, the author examined the expression patterns of the critical transcription factors mRNAs during the differentiation of preadipocytes in stromal vascular (SV) cells derived from bovine adipose tissue (Chapter 4). C/EBPβ mRNA in the bovine SV cells was stably expressed throughout the differentiation period. Additionally, the relatively high expression of PPARγ was observed before the induction of differentiation. On the other hand, C/EBPα mRNA level was extremely low before the induction of differentiation. These results suggested that bovine SV cells derived from adipose tissue contained the preadipocytes reaching at more advanced stage of differentiation than 3T3-L1 preadipocytes did. Despite the different expression patterns of these transcription factors, myostatin suppressed the increase in PPARγ mRNA expression and the induction of C/EBPα mRNA without affecting the expression of C/EBPβ mRNA in the early phase of differentiation, which was similar to the effect of myostatin on 3T3-L1 preadipocyte differentiation. Furthermore, the author revealed that follistatin interfered with myostatin action during bovine preadipocyte differentiation when the cells were co-treated with myostatin and follistatin.

Activin, a multifunctional growth and differentiation factor that belongs to the TGF-β superfamily, is homodimer of activin β$_A$ subunit. Recently, the expressions of activin β$_A$ and activin receptor mRNAs were found in many tissues throughout the body including muscle and fat. Activin A was reported to act on many cell types, however, its action on preadipocyte differentiation was not still investigated. In Chapter 5, the author investigated the effect of activin A on the differentiation of bovine and 3T3-L1 preadipocytes. Activin A inhibited the differentiation of bovine and 3T3-L1 preadipocytes by reducing PPARγ and C/EBPα mRNA expression but not affecting C/EBPβ expression, which was similar to the effect of myostatin on the preadipocyte differentiation. In Section 2 in Chapter 5, the author investigated the further mechanism that activin A inhibited the differentiation of 3T3-L1 preadipocyte. Activin A inhibited the DNA binding activity of C/EBPβ, which was considered to suppress the preadipocyte differentiation. Activin A possibly prevented adipogenesis in the early phase via Smad3-mediated impairment of the DNA binding ability of C/EBPβ, and thus of the transactivation function of C/EBPβ.

In Section 1 in Chapter 5, the author revealed that follistatin reversed the inhibitory effect of activin A on bovine preadipocyte differentiation. Additionally, the treatment with follistatin alone increased GPDH activity, which suggested that bovine SV cells in the present study may produce substantial level of activin A and/or myostatin to inhibit preadipocyte differentiation, and thus exogenous follistatin promoted adipogenesis.

The expression of activin A and follistatin is widely distributed in gonadal and non-gonadal tissues including muscular and adipose tissues. Myostatin is found

primarily in skeletal muscle, and is also detected in adipose tissue. Therefore, myostatin and/or activin A secreted from muscular and/or adipose tissues possibly inhibit the differentiation of preadipocytes within muscle, and follistatin secreted from these tissues may suppress the inhibitory effect of myostatin and activin A. The physiological balance of these paracrine/autocrine factors possibly regulates preadipocyte differentiation in muscle.

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