Involvement of Cytoskeleton-associated Proteins in the Commitment of C3H10T1/2 Pluripotent Stem Cells to Adipocyte Lineage Induced by BMP2/4*

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The developmental pathway that gives rise to mature adipocytes involves two distinct stages: commitment and terminal differentiation. Although the important proteins/factors contributing to terminal adipocyte differentiation have been well defined, the proteins/factors in the commitment of mesenchymal stem cells to the adipocyte lineage cells have not. In this study, we applied proteomics analysis profiling to characterize differences between uncommitted C3H10T1/2 pluripotent stem cells and those that have been committed to the adipocyte lineage by BMP4 or BMP2 with the goal to identify such proteins/factors and to understand the molecular mechanisms that govern the earliest stages of adipocyte lineage commitment. Eight proteins were found to be up-regulated by BMP2, and 27 proteins were up-regulated by BMP4, whereas five unique proteins were up-regulated at least 10-fold by both BMP2/4, including three cytoskeleton-associated proteins (e.g., lysyl oxidase (LOX), translationally controlled tumor protein 1 (TPT1), and αB-crystallin). Western blotting further confirmed the induction of the expression of these cytoskeleton-associated proteins in the committed C3H10T1/2 induced by BMP2/4. Importantly, knockdown of LOX expression totally prevented the commitment, whereas knockdown of TPT1 and αB-crystallin expression partially inhibited the commitment. Several published reports suggest that cell shape can influence the differentiation of partially committed precursors of adipocytes, osteoblasts, and chondrocytes. We observed a dramatic change of cell shape during the commitment process, and we showed that knockdown of these cytoskeleton-associated proteins prevented the cell shape change and restored F-actin organization into stress fibers and inhibited the commitment to the adipocyte lineage. Our studies indicate that these differentially expressed cytoskeleton-associate proteins might determine the fate of mesenchymal stem cells to commit to the adipocyte lineage through cell shape regulation. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.002691, 1–8, 2011.

Obesity results when caloric intake exceeds energy expenditure, leading to adipocyte hypertrophy and hyperplasia, including the recruitment of stem cells and subsequent differentiation of stromal-vascular preadipocytes (1–5). The stromal-vascular preadipocyte arises from a multipotent stem cell population of mesodermal origin. These mesenchymal stem cells (MSCs)1 have the capacity to commit to several distinct cell types, including adipocytes, myoblasts, osteoblasts, and chondrocytes (6–8). The genes that are involved in the earliest stages of myoblast (MyoD) (9, 10), chondroblast (Sox9) (11, 12), and osteoblast (Runx2/Cbfa1 and osterix) (13–16) lineage determination by MSCs have already been identified. However, the genes governing the earliest stages of adipocyte determination have not yet been identified.

Programming the adipose lineage is a multistep process comprising an initial commitment step in which cells become restricted to the adipocyte lineage but do not yet express markers of terminal differentiation and subsequent activation of a network of transcription factors resulting in the adipocyte phenotype (17). Although the important proteins that contribute to terminal adipocyte differentiation have been well defined (18–20), the proteins involved in commitment of pluripotent stem cells to the adipocyte lineage have not. However,

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1 The abbreviations used are: MSC, mesenchymal stem cell; 2-DE, two-dimensional gel electrophoresis; BMP, bone morphogenetic protein; LOX, lysyl oxidase; TPT1, translationally controlled tumor protein 1; MyoD, myogenic differentiation antigen; Sox9, SRY-related high mobility group box gene 9; Runx2, Runt-related transcription factor 2; Cbfa1, core binding factor α1; 422/aP2, fatty acid-binding protein; PMF, peptide mass fingerprinting; NCBI, National Center for Biotechnology Information.
to understand the processes that occur during adipocyte commitment, a multipotent stem cell line is needed. The C3H10T1/2 stem cell line was originally isolated from C3H mouse embryos (21) and behaves similarly to mesenchymal stem cells, making this cell line ideal for studying factors involved in the adipocyte commitment process. Our previous findings indicate that bone morphogenetic protein (BMP) 2/4 treatment of C3H10T1/2 cells induces nearly complete commitment to the adipocyte lineage (22–24). These findings should be beneficial in unraveling the processes involved in adipose lineage commitment.

In this study, we applied proteomics analysis profiling to characterize differences between uncommitted C3H10T1/2 cells and those that have been committed by BMP4 or BMP2 with the goal to identify adipocyte lineage commitment factors. Eight proteins were found to be up-regulated by BMP2, and 27 proteins were up-regulated by BMP4, whereas five unique proteins were up-regulated at least 10-fold by both BMP2 and BMP4, among which three proteins are cytoskeleton-associated proteins. Studies have demonstrated the importance of both cell shape and extracellular matrix remodeling during the course of adipose commitment and development (25, 26). Our studies indicate that cytoskeleton-associated protein lysyl oxidase (LOX), translationally controlled tumor protein 1 (TPT1), and α-B-crystallin are elevated dramatically with BMP4 or BMP2 treatment. This study describes the characterization of LOX, TPT1, and α-B-crystallin during preadipocyte commitment of 10T1/2 cells and proposes a role for these proteins during the adipocyte commitment process.

EXPERIMENTAL PROCEDURES

Cell Culture and Induction of Commitment/Differentiation—To induce adipocyte lineage commitment, C3H10T1/2 stem cells were plated at low density and cultured in DMEM containing 10% calf serum without or with purified recombinant BMP2 (50 ng/ml) or BMP4 (10 ng/ml). To induce differentiation, 2-day postconfluent cells were fed DMEM containing 10% fetal bovine serum (FBS), 1 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine for 2 days and then fed DMEM with 10% FBS and 1 μg/ml insulin for another 2 days after which they were cultured in DMEM with 10% FBS.

Oil Red O Staining—C3H10T1/2 stem cells treated with or without StealthTM siRNA were induced to differentiate as described above. On day 8, the cells were washed three times with phosphate-buffered saline (PBS) and then fixed for 10 min with 3.7% formaldehyde. Oil red O (0.5% in isopropanol) was diluted with water (3:2), filtered through a 0.45-μm filter, and incubated with the fixed cells for 1 h at room temperature. After that, the cells were washed with water, and the stained fat droplets in the adipocytes were visualized by light microscopy and photographed.

Western Blotting—Cells were washed with cold PBS (pH 7.4) and then scraped into lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, phosphatase inhibitors (10 mM Na2VO4 and 10 mM NaF), and protease inhibitor mixture (Roche Applied Science). Lysates were heated at 100 °C for 10 min and clarified by centrifugation. Equal amounts of protein were subjected to SDS-PAGE and immunoblotted with specific primary antibodies. 422/αP2 antibody was from Dr. M. Daniel Lane’s laboratory; Smad4 antibody was purchased from Abcam (Cambridge, UK); p38 MAPK kinase antibody was from Cell Signaling Technology (Beverly, MA); LOX, TPT1, and α-B-crystallin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); and β-actin antibody was from Sigma-Aldrich.

RNA Interference—Stealth siRNA duplexes specific for Smad4, p38 MAPK, lysyl oxidase, α-B-crystallin, and Tpt1 were designed and synthesized by Invitrogen. The silencing effects of several siRNA duplexes were screened and tested initially for their ability to knock down expression of target genes by Western blotting. The sequences for successful RNAi knockdown were as follows: CAUAACACCUAAUUGCCUCACCACCA for Smad4, CCUUUGAAAGGAGGACCUUCUCAUA for p38 MAPK, GGGGAUGUGAGACGCUAGACCA for lysyl oxidase, UGAGGACUCUAAAGACGAGGGA for α-B-crystallin, and UGACUGACGCAGGGCGAUAUAU for Tpt1. Stealth siRNA negative control duplexes with a similar GC content were used as control. C3H10T1/2 stem cells were transfected at 30–50% confluence with siRNA duplexes by using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer’s instructions.

Immunofluorescence—C3H10T1/2 cells were plated on the coverslips and treated as described above; postconfluence, cells were washed three times with PBS and fixed in 4% (w/v) formaldehyde for 10 min at room temperature. Then the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked with 10% fetal bovine serum in PBS for 2 h. F-actin was stained by using rhodamine-conjugated phalloidin (Molecular Probes, Eugene OR). Images were captured by using a Zeiss LSM 510 confocal microscope.

2-DE Analysis—C3H10T1/2 cells were treated with or without BMP2/4 as described above; cell lysates were prepared according to the manufacturer’s instructions. Isoelectric focusing was performed using an IPGphor unit (Amersham Biosciences) with precast nonlinear IPG gel strips (17 cm, pH 3–11; Amersham Biosciences). 300 μg of total proteins were mixed with rehydration solution (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 50 mM DTT, and a trace amount of bromophenol blue) in a final volume of 350 μl and incubated for 12 h at room temperature before separation by IEF at 500 V for 1 h, 1,000 V for 1 h, 2,000 V for 1 h, 4,000 V for 1 h, or 8,000 V for 5 h (50 mA/gel strip). The gel strips were then immediately equilibrated in equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, and 2% w/v SDS). Separation in the second dimension was carried out using 12% SDS-PAGE followed by electrophoresis in a Protean II xi 2-D cell (Bio-Rad) at 20 mA for the first 20 min and then at 40 mA until the bromophenol blue reached the bottom of the gel. The procedure was repeated three times for each sample to ensure reproducibility.

Staining of Two-dimensional Gels—The two-dimensional gels were stained using a silver staining kit (Amersham Biosciences). Briefly, the gels were fixed in 40% ethanol and 10% acetic acid for 30 min; sensitized in a solution of 25% (w/v) ethanol glutaraldehyde, 5% (w/v) sodium thiosulfate, and 6.8% sodium acetate for 30 min; and washed three times with water for 15 min each. The gels were subsequently immersed in 2.5% (w/v) silver nitrate and 37% (w/v) formaldehyde for 20 min and developed in a mixture of 6.25 g of sodium carbonate and 37% (w/v) formaldehyde for 2–5 min, and the reaction was then stopped in EDTA-Na2·2H2O.

In-gel Digestion—Protein spots were excised from the gels and placed into a 96-well microtiter plate. Gel pieces were destained with a solution of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate (1:1) for 20 min at room temperature. Then they were washed twice with deionized water and shrunk by dehydration in ACN. The samples were then swollen in a digestion buffer containing 20 mM ammonium bicarbonate and 12.5 ng/μl trypsin at 4 °C. After a 30-min incubation, the gels were digested more than 12 h at 37 °C. Peptides were then extracted twice using 0.1% TFA in 50% ACN.

Mass Spectrometry and Database Search—The peptide extracts were dried under the protection of N2. For MALDI-TOF-MS, the
peptides were eluted with 0.7 μl of matrix solution (α-cyano-4-hydroxycinnamic acid in 0.1% TFA and 50% ACN). Samples were allowed to air dry before mass spectrometry. All mass spectra were acquired in a positive ion mode by a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA). The instrument was operated at an accelerating voltage of 20 kV. A 200-Hz pulsed neodymium-doped yttrium aluminium garnet laser (355 nm) was used for MALDI. Proteolytic peptides of the standard myoglobin with known molecular masses were used for calibration. All peptide mass fingerprinting (PMF) spectra were taken from signal averaging of 1000 laser shots. The five strongest peaks in each PMF spectrum were selected to perform tandem mass spectrometry with averaging of 1500 laser shots. The laser intensity was kept constant for all of the samples. Data from MALDI-TOF/TOF-MS were analyzed using GPS Explorer (Applied Biosystems; version 3.6) as the peak list generator and Mascot (Matrix Science, London, UK; version 2.1) as the search software. The following parameters were used in the search: Mus musculus, protein molecular mass ranged from 700 to 3200 Da, trypsin digestion with one missing cleavage, peptide tolerance of 0.2 Da, MS/MS tolerance of 0.6 Da, and possible oxidation of methionine. The NCBI database (version March 16, 2007) was used. The number of protein entries in the database actually searched was 4,182,491 in total (162,314 M. musculus). There was no fixed modification.

PMF Acceptance Criteria—If the ion score was equal to or greater than the Mascot significance level calculated for the search, the peptide identification was considered to be statistically non-random at the 95% confidence interval.

RESULTS

Commitment of Pluripotent Stem Cells to Adipocyte Lineage by BMP2 or BMP4—As previously reported, BMP4 can induce the commitment of C3H10T1/2 stem cells into cells that possess the characteristics of preadipocytes (24). BMP2, a homologue of BMP4, can also induce the commitment although at a slightly higher concentration (23). To continue this study, the experiment was repeated: as illustrated in Fig. 1, treatment with both a higher level of BMP2 (50 ng/ml) or a lower level of BMP4 (10 ng/ml) could induce commitment/differentiation of C3H10T1/2 pluripotent stem cells equally effectively as assessed by cytoplasmic triglyceride accumulation (Fig. 1A) and the expression of 422/aP2 (Fig. 1B). This established cell model was used in the following experiments to unravel the processes involved in the adipocyte lineage commitment.

Proteomics Analysis of Differentially Expressed Proteins Induced by BMP2/4 during Adipocyte Lineage Commitment—The human BMP2 has a high homology (92%) with BMP4. Although investigators have suggested differences in the effects of BMP2 and BMP4 on a variety of cell types (27–32), the similar effect of these two factors on the commitment of stem cells to preadipocytes allowed us to identify collectively expressed proteins induced by BMP2/4 that may contribute to adipocyte lineage commitment. Proteomics analysis was performed to identify target proteins during the commitment induced by BMP2/4. Proteins from cells extracts (induced or not with BMP) were separated by 2-DE and visualized by silver nitrate staining (Fig. 2A). Approximately 1000 protein spots were mapped in each of the 2-DE gels. Proteins were considered differentially expressed between committed and uncommitted cells when the magnitude of difference was greater than 10-fold or more and the result was repeated three times. Eight proteins were found to be up-regulated by BMP2, and 27 proteins were up-regulated by BMP4, whereas five proteins were up-regulated by both BMP2 and BMP4. Tables I and II summarize the proteins up-regulated during the commitment induced by BMP2 and BMP4, respectively. Among the five proteins up-regulated by both BMP2 and BMP4, three are cytoskeleton-associated proteins, i.e. LOX, TPT1, and αB-crystallin. Enlarged images of these three differentially expressed protein spots are shown in Fig. 2B, and the induction of LOX, TPT1, and αB-crystallin was further confirmed by Western blotting (Fig. 2C).

Lox, Tpt1, and αB-Crystallin Are Downstream Target Genes of BMP Signaling Pathway—BMP/Smad and BMP/p38 MAPK are two signaling pathways involved in the BMP responses in a variety of cell types. Our previous studies indicated that the BMP/Smad signaling pathway is required for BMP-induced adipocyte lineage commitment of C3H10T1/2 stem cells, whereas BMP/p38 MAPK is only partially involved in the commitment, and the effect of its disruption is lesser than that of BMP/Smad.
To investigate whether the differentially expressed proteins identified above are downstream target genes of BMP signaling pathways, the Smad4/p38 MAPK RNAi knockdown experiments were repeated, and the effects on the expression of the identified differentially expressed proteins were examined. We found that Smad4 RNAi inhibited LOX and αB-crystallin expression, whereas knockdown of p38 MAPK had a slight inhibitory effect on LOX and αB-crystallin expression, suggesting a more vital role for BMP/Smad in the induction of these two proteins. For TPT1, both Smad4 and p38 MAPK RNAi could down-regulate its expression equally efficiently, whereas Smad4 RNAi combined with p38 MAPK RNAi could almost totally block the expression of TPT1 (Fig. 3). Our results indicated that LOX, TPT1, and αB-crystallin are target proteins of the BMP signaling pathway.

Knockdown of LOX, TPT1, and αB-Crystallin Expression Prevented Commitment of C3H10T1/2 Pluripotent Stem Cells to Adipocyte Lineage Induced by BMP2/4—To further ascertain whether LOX, TPT1, and αB-crystallin are required for the adipocyte lineage commitment process, the expression of these proteins was knocked down in proliferating C3H10T1/2 cells with siRNA with BMP treatment. Compared with the control cells that were transfected with non-target-directed siRNA, the expression of these proteins was significantly reduced by using siRNA as demonstrated by Western blotting (Fig. 4A). After reaching postconfluence, the cells were subjected to a standard adipocyte differentiation protocol, and it was found that LOX knockdown abolished the acquisition of the adipocyte phenotype as determined by the accumulation of cytoplasmic triglyceride stained with oil red O (Fig. 4B) and adipocyte marker gene (422/aP2) expression (Fig. 4C). Knockdown of αB-crystallin or TPT1 had only a ~50% inhibitory effect on the BMP-induced commitment of C3H10T1/2 stem cells (Fig. 4, B and C). These findings suggested that LOX, TPT1, and αB-crystallin all contributed to BMP-induced adipocyte lineage commitment of C3H10T1/2 stem cells, whereas LOX plays a more important role for the adipocyte commitment. Our results confirmed that these three cytoskeleton-associated proteins are involved in the commitment of C3H10T1/2 stem cells to preadipocytes.

Knockdown of LOX, TPT1, and αB-Crystallin Expression Reorganize BMP4-induced F-actin Disruption during Commitment of C3H10T1/2 to Preadipocytes—It has been indicated that cell shape is involved in adipose commitment and development (25, 33, 34), and the cytoskeleton plays important roles in determining/maintaining cell shape (35, 36). Because the three differentially expressed proteins are cytoskeleton-associated proteins, we further investigated whether the induction of these proteins was associated with a cell shape change during the commitment process. We found that the postconfluent uncommitted C3H10T1/2 cells have an extended broad and flat cytoplasm (Fig. 5A) and that the F-actin filaments in these uncommitted MSCs are present in the form of stress fibers, forming long linear

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**Fig. 2.** Proteomics analysis and validation of differentially expressed proteins induced by BMP2/4. A, 2-DE analysis of differentially expressed proteins induced by BMP2/4. Proteins (300 μg) from cells treated with or without BMP2/4 were analyzed by 2-DE. The first dimension was 17-cm, pH 3–10 IPG, and the second dimension was 12% SDS-PAGE. Proteins in the gels were visualized by silver nitrate staining. Proteins showing differential expression were isolated and identified using MALDI-MS and MALDI-MS/MS. B, enlarged images of differentially expressed protein spots between untreated and BMP2/4-treated C3H10T1/2 cells. Selected regions of 2-DE gels illustrate differentially expressed proteins among untreated C3H101/2 and BMP2- or BMP4-treated C3H101/2. Spots of interest are indicated with arrows. C, verification of the differentially expressed proteins in BMP2/4-treated cells. Western blot analyses showed that proteins were up-regulated in the BMP2/4-treated C3H101/2 cells. The differentially expressed proteins LOX, TPT1, and αB-crystallin are more abundant in BMP2/4-treated cells than in untreated cells. β-A ctin was used as loading control.
After treatment with BMP2/4, the postconfluent committed C3H10T1/2 cells had higher cell density (the cell number increased ~1.5-fold), their shape changed from spindle to ovoid or round when reaching confluence (Fig. 5A), and in parallel to this shape transition, the stress fibers of F-actin were significantly decreased after the induction with BMP2/4. The

**Table I**

| Spot no. | Protein name                          | NCBI accession no. | Theoretical molecular mass | Theoretical pl | Sequence coverage | Matched/searched |
|----------|---------------------------------------|--------------------|-----------------------------|----------------|------------------|------------------|
| 1        | Crystallin, αB                        | gi|6753530                | 20,056.4                   | 6.76            | 65               | 13/127           |
| 2        | Protein-lysine 6-oxidase precursor    | gi|126638                 | 46,671.5                   | 8.73            | 17               | 5/148            |
| 3        | Tumor protein, translationally controlled 1 | gi|6678437                | 19,449.6                   | 4.76            | 53               | 9/98             |
| 4        | SH3-binding domain glutamic acid-rich protein-like | gi|9910548                | 12,803.2                   | 4.99            | 50               | 5/169            |
| 5        | SH3 domain protein 3                  | gi|22267440               | 23,767.9                   | 5.46            | 50               | 11/100           |
| 6        | High density lipoprotein-binding protein | gi|19527028               | 141,654.3                  | 6.43            | 22               | 27/193           |
| 7        | Macrophage migration-inhibitory factor | gi|6754696                 | 12,496.2                   | 6.79            | 42               | 4/76             |
| 8        | UMP-CMP kinase                        | gi|23821758               | 22,151.3                   | 5.68            | 66               | 15/127           |

**Table II**

| Spot no. | Protein name                          | NCBI accession no. | Theoretical molecular mass | Theoretical pl | Sequence coverage | Matched/searched |
|----------|---------------------------------------|--------------------|-----------------------------|----------------|------------------|------------------|
| 1        | Crystallin, αB                        | gi|6753530                | 20,056.4                   | 6.76            | 65               | 13/127           |
| 2        | Protein-lysine 6-oxidase precursor    | gi|126638                 | 46,671.5                   | 8.73            | 17               | 5/148            |
| 3        | Tumor protein, translationally controlled 1 | gi|6678437                | 19,449.6                   | 4.76            | 53               | 9/98             |
| 4        | SH3-binding domain glutamic acid-rich protein-like | gi|9910548                | 12,803.2                   | 4.99            | 50               | 5/169            |
| 5        | SH3 domain protein 3                  | gi|22267440               | 23,767.9                   | 5.46            | 50               | 11/100           |
| 6        | High density lipoprotein-binding protein | gi|19527028               | 141,654.3                  | 6.43            | 22               | 27/193           |
| 7        | Macrophage migration-inhibitory factor | gi|6754696                 | 12,496.2                   | 6.79            | 42               | 4/76             |
| 8        | UMP-CMP kinase                        | gi|23821758               | 22,151.3                   | 5.68            | 66               | 15/127           |

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organization of F-actin was disrupted or altered, resulting in filament clumping or stacking in little arrays around the cell periphery (Fig. 5B).

The knockdown of LOX expression by siRNA could almost totally rescue the cell shape change (Fig. 5A) and reorganized the stress fibers of F-actin filaments, which were disrupted during commitment induced by BMP2/4 (Fig. 5B), and consequently the commitment induced by BMP was almost totally prevented after LOX knockdown (Fig. 4, B and C). We also found that knocked down expression of αB-crystallin or TPT1 by siRNA could only partially rescue the stress fibers of F-actin filaments, and the commitment was only partially inhibited (Fig. 4). These findings indicated that the change of cell shape may be one of the mechanisms involved in adipocyte commitment induced by BMP.

**DISCUSSION**

Although the sequential steps of terminal adipocyte differentiation have been clearly defined (17, 37), the steps and the mechanism in the commitment of pluripotent stem cells to the adipocyte lineage have not been well elucidated. The C3H10T1/2 pluripotent stem cell line behaves similarly to mesenchymal stem cells, which have the potential to develop into osteoblasts, chondrocytes, and adipocytes (38–41). Our previous findings indicated that BMP2/4 treatment of C3H10T1/2 cells induces nearly complete commitment and subsequent differentiation to the adipocyte lineage (23, 24). These established adipocyte lineage commitment cell models have made it possible to unravel the mechanism involved in the commitment process.

In this study, proteomics analysis profiling was used to identify differentially expressed proteins between uncommitted C3H10T1/2 cells and those that have been committed by BMP2/4 with the goal to identify adipocyte lineage commitment proteins. Proteomics analysis showed that LOX, TPT1, and αB-crystallin were highly expressed in committed cells compared with uncommitted cells (Fig. 2), indicating potential roles in adipocyte lineage commitment. Functional studies indicated that LOX had a critical role during adipocyte lineage commitment with the evidence that LOX knockdown can almost totally block commitment (Fig. 4). However, TPT1 and αB-crystallin were partially involved in the commitment: TPT1 and αB-crystallin knockdown could only partially inhibit the commitment (Fig. 4). Further evidence indicates that all three of these proteins are downstream target genes for the BMP signaling pathway (Fig. 3), although there is no evidence showing that Tpt1 and αB-crystallin are the direct target genes of BMP signaling pathway. C3H10T1/2 stem cells were plated at 30% confluence, transfected with Smad4 or p38 MAPK Stealth RNAi, and 24 h later treated with BMP4 until 2 days postconfluent. Knocked down expression of Smad4 or p38 MAPK and its effect on the expression of the differentially expressed proteins (LOX, TPT1, and αB-crystallin) were confirmed by immunoblotting.

**Fig. 3.** LOx, Tpt1, and αB-crystallin are downstream target genes of BMP signaling pathway. C3H10T1/2 stem cells were plated at 30% confluence, transfected with Smad4 or p38 MAPK Stealth RNAi, and 24 h later treated with BMP4 until 2 days postconfluent. Knocked down expression of Smad4 or p38 MAPK and its effect on the expression of the differentially expressed proteins (LOX, TPT1, and αB-crystallin) were confirmed by immunoblotting.

**Fig. 4.** LOX, TPT1, and αB-crystallin are required for adipocyte lineage commitment of C3H10T1/2 pluripotent stem cells. C3H10T1/2 stem cells were plated at 30% confluence and transfected with LOX, TPT1, and αB-crystallin Stealth RNAi 24 h later. Upon reaching confluence, knocked down expression of LOX, TPT1, and αB-crystallin was verified by Western blotting. β-Actin served as loading control. The effect on the adipocyte lineage commitment and subsequent differentiation was assessed both by oil red O staining (B) and 422/αP2 expression (C). MDI: adipocyte differentiation cocktail, 1 μM dexamethasone, 1 μg/ml insulin and 0.5 mM isobutylmethyl-xanthine.
cytes, myoblasts, and osteoblasts from MSCs through regulating the expression of their master regulators (44). It has been shown that BMPs can inhibit myogenic differentiation but promote the differentiation of mesenchymal stem cells into osteoblasts, and further studies indicate that BMP2 can dramatically increase Runx2 expression and induce osteoblast-specific gene expression in C2C12 myoblasts (45). BMP2 inhibits the differentiation of C2C12 myoblasts and myogenic transcription factor-introduced C3H10T1/2 cells into mature myotubes by suppressing the transcriptional activity of myogenic factors such as MyoD and myogenin (46, 47). Although the roles of BMPs in adipose development are not well understood, a recent study reported that mice lacking the zinc finger protein Schnurri-2, which is regulated by BMP2, exhibited a significant reduction in white fat mass (48), and in this study, we report that BMP2/4 can induce the expression of LOX, TPT1, and αB-crystallin, which contribute to the adipocyte lineage commitment from C3H10T1/2 pluripotent stem cells.

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