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Oscillatory Expression of Stem Cell-associated Genes in Pattern Forming Calcifying Vascular Cells

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Oscillatory Expression of Stem Cell-associated Genes in Pattern Forming Calcifying Vascular Cells

A thesis submitted in partial satisfaction

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in Biomedical Engineering

by

Anthony H. Wang

2012

ABSTRACT OF THE THESIS

Oscillatory Expression of Stem Cell-associated Genes

in Pattern Forming Calcifying Vascular Cells

by

Anthony H. Wang

Master of Science in Biomedical Engineering

University of California, Los Angeles, 2012

Professor Alan Garfinkel, Co-Chair

Professor Kristina I. Boström, Co-Chair

Multipotent CVCs are used as models for atherosclerotic lesion cells due to their capacity to calcify, but the molecular or cellular mechanisms involved are not completely known. Recent work has reported that stem cell-associated factors show patterned responses in vertebrate segmentation and other cells in embryonic development, suggesting that oscillatory expression is a general feature for many cellular events. In this study, we explored connections between the expression of multipotency markers and pattern formations in CVCs. Control CVCs, BMP-4 treated CVCs, and MGP depleted CVCs were cultured separately in a two-week time period, and the expression of Sox2, Nanog, Klf4, and Oct-3/4 were analyzed with real-time PCR. Furthermore, the relationship between the osteogenic potential of CVC and these genes was

briefly explored. Our results suggest that CVCs may exhibit oscillatory genetic expression as they form patterns, and that BMP inhibition by MGP is essential to maintaining oscillations and pattern formations.

The thesis of Anthony H. Wang is approved.

Ichiro Nishimura

Alan Garfinkel, Committee Co-Chair

Kristina I. Boström, Committee Co-Chair

University of California, Los Angeles

2012

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INTRODUCTION

In embryonic development, mesenchymal stem cells organize and develop patterned tissues. In vascular diseases, such as atherosclerosis and vascular calcification, such embryonic events have been observed, as vascular mesenchymal cells (VMCs, also referred to as calcifying vascular cells, CVCs), subpopulation of cells from bovine or human artery wall, differentiate into osteoblasts and other cell types[1]. These multipotent cells have the capacity for self-renewal and differentiation along different lineages spontaneously and with induction media. Due to their multipotentiality, CVCs have been used as a model for atherosclerotic lesion cells that have the capacity to calcify, but the molecular or cellular mechanisms involved are not completely known.

Understanding the multipotentiality of CVC model would advance our knowledge of vascular development as it occurs in embryogenesis and wound healing. Expression of stem cells-associated genes, including SRY (sex determining region Y)-box 2 (Sox2), homeobox protein Nanog, Kruppel-like factor 4 (Klf4) and octamer-binding transcription factor-3/4 (Oct-3/4), has been used as stem cell markers for identification of multipotent cells. Sox2 is a crucial transcriptional regulator associated with multipotent and unipotent stem cell, and Nanog, Klf4, and Oct-3/4 have been identified as involved in pluripotency of cells[2,3]. They are important to maintain self-renewal of undifferentiated embryonic stem cells. Studies have reported that several so-called stemness factors show patterned response in other cells, suggesting that oscillatory expression is a general feature for many cellular events[4-6] and may also occur in CVCs.

Previous studies have found that CVCs progressively form patterns in which osteogenic and chondrogenic differentiation occurs[1,7]. CVCs express bone morphogenetic protein (BMP)-

2/4, potent osteoinductive factors[8] that can promote the calcification in CVCs, and Matrix GLA protein (MGP), an inhibitor of BMP-2/4 that limits calcification[9,10]. Condensations, nodule size and pattern formation in CVCs are affected by the relative expression and interaction of BMP and MGP, a finding that is supported by a mathematical model based on molecular morphogens reaction-diffusion process[11].

BMP-4 is a member of the TGF-β superfamily of growth factors[12], and is known to stimulate vasculogenesis and angiogenesis[8]. It is essential for induction of mesoderm and endothelial progenitor cell differentiation[13]. BMPs are involved in promoting nodule formation and size in CVCs[14,15]. In previous studies, we have identified a regulatory pathway which BMP-4, along with BMP-2, activates expression of MGP and VEGF[8,16,17]. The pathway suggests that a balance between BMP and MGP is important to maintain cellular characteristics in CVCs.

MGP is a small protein expressed in endothelial cell (EC)[18-20] and is considered to be a calcification inhibitor based on the extensive arterial calcification observed in MGP null mice[21]. It modulates local BMP activity by antagonizing BMP-2, -4, and -7[16,22], and inhibits angiogenesis through feedback inhibition of BMP-4[23,24]. MGP is also induced in pattern formation and limits nodule size in CVCs[9,11], which is essential in arterial calcification. Absence of MGP increases overall vascular BMP activity, recruiting in both arterial calcification and arteriovenous malformations (AVMs)[22].

In this study, we explore the relationship between stem cell-associated gene expression (Sox2, Nanog, Klf4, and Oct-3/4) and pattern formation in CVCs. Results showed that expression of stemness markers oscillate as CVCs progressively developed swirling patterns after confluency. The largest oscillations of expression were demonstrated for Sox2 and Nanog and were further studied as we tested the effects of BMP-4 and MGP. Overall, BMP-4 had minor

effects on Sox2 and Nanog expression. By contrast, the depletion of MGP disrupted the oscillations in gene expression and the changed pattern formation in CVCs. Together, our results suggest that expression of stem cell-associated genes oscillate in CVCs as they form patterns, and that BMP-inhibition by MGP is essential to maintain the oscillations and the pattern formation.

MATERIALS AND METHODS

Cell Culture

Bovine aortic smooth muscle cells (BASMCs) were harvested, cultured, and passaged from explants, and CVCs were subcloned from these cells by dilutional cloning as previously described[1,7,25,26]. CVCs that exhibited the most multilineage potential were selected for experiments and were used at passage 2 to 6 after subcloning in order to ensure maintained multilineage potential and sufficient efficiency of siRNA transfection. Cells were cultured in Dulbecco's Modified Eagle Medium with 4.5 g/L glucose (Invitrogen, Carlsbad, CA) containing 10% (v/v) heat-inactivated FBS (Hyclone Labs, Novato, CA) and supplemented with 1% (v/v) penicillin-streptomycin (Invitrogen) at 37ºC, 5% CO2 in humidified incubators. Culture media were changed every 3 to 4 days until testing. For experiments, CVC were plated at approximately 70-90% confluence.

siRNA Transfections

Transfections of CVC were performed by electroporation using the Amaxa Nucleofector® (Lonza, Allendale, NJ) and the human AoSMC Nucleofector® kit (Lonza). The number of cells per electroporation and Nucleofector settings was optimized to 0.5-1x10^6 cells as per manufacturer's instructions. 60 nM of siRNA was used per well in 6-well culture plates as previously described[8]. Two siRNAs to bovine MGP (Silencer® predesigned siRNA, Ambion, NY) and scrambled siRNA with the same nucleotide content were tested. Transfected cells were incubated in humidified incubators for a period of 24 or 48 hours. RNA analysis was performed

to determine transfection efficiency. The siRNA that provided the most efficient inhibition (>90-95%) was used for experiments.

Long-term Culture

Control CVCs and transfected cells were cultured separately in 12-well plates, and cell medium was not renewed during the course of experiment. For treatments, 40 ng/ml human recombinant BMP-4 (R&D Systems, Minneapolis, MN) was added to the medium immediately after cells were plated. RNA was isolated from one well of control CVCs, transfected CVCs, and treated CVCs respectively, every 48 hours for a period of two weeks, after which RNA analysis was performed. Cells were maintained and cultured for 14 days until pattern formation of CVC was observed.

RNA Analysis

Total RNA was isolated from cultured cells using RNeasy kit (Qiagen, Valencia, CA). Real-time PCR assays were performed as previously described[27,28], and bovine (b) glyceraldehyde 3-phosphate dehydrogenase (bGAPDH) was used as control gene[28]. The primer and probes for bSOX2, bovine bOCT3/4, bNANOG, bKlf4, and bMGP were obtained from Applied Biosystems as part of Taqman Gene Expression Assay.

CVC Osteogenic Differentiation

So-called Osteocyte differentiation basal medium was supplemented with 5 mM beta-glycerophosphate (Invitrogen). This supplement is optional for osteoblastic differentiation in CVCs and accelerates mineralization as previously described[1].

CVCs were passaged and cultured in 6-well plates for one week without changing the culture medium. On Day 7, culture medium was removed. Cells were rinsed with 1X phosphate-buffered saline (PBS) (Irvine Scientific) and were cultured with osteocyte differentiation basal medium in humidified incubators at 37 ºC for 3 days. On Day 10, alkaline phosphatase histochemical (AP) staining, calcium assay, and enzyme-linked immunosorbent assay (ELISA) were performed. ELISA was performed to confirm the presence of proteins prior to the other assays. Calcium concentrations were analyzed using QuantiChrom^{TM} Calcium Assay Kit (BioAssay Systems, Hayward, CA) following the manufacturer's protocol, and protein concentration was measured using Bio-Rad Protein Assay (Bio-Rad, Hercule, CA).

AP Histochemical Staining

AP buffer was prepared by mixing of 100 mM of Tris-HCl (pH 9.5), 100 mM of NaCl, and 50 mM of $MgCl_2$ in deionized water. AP staining solution was prepared by mixing 200 μL of NBT/BCIP stock solution (Boehringer-Mannheim, Indianapolis, IN) in 10 ml of prepared buffer. Cells were rinsed with buffer mixture 3 times, and staining solution was added. The 6-well plates were then incubated in the dark at 37ºC until color developed (30 minutes to 24 hours). The reaction was stopped by aspirating the staining solution followed by addition of 1X Tris-EDTA solution (pH 8.0), and the cells were fixed in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) for 5 minutes at room temperature.

ELISA

Cells were rinsed with 1X PBS, and cell lysis were collected with 500 μL of 1X PBS. 190 μL of p-Nitrophenyl Phosphate Liquid substrate system (Sigma-Aldrich, St. Louis, MO) was added to

10 μL of Cell lysates in 96-well plates, and the analysis was performed by following manufacturer's protocol.

RESULTS

CVCs exhibit a higher expression of stem cell-associated gene expression

In previous studies, CVCs, derived by dilutional cloning of BASMCs, showed that they have potential to form patterns and undergo osteoblastic differentiation and mineralization[1]. To study the expression of undifferentiated stem cell markers in CVC, we selected a CVC clone that formed pattern and had osteogenic potential. It had been derived from a single pericyte-like cell (Figure 1A) by previously described methods[7,25,26]. To determine if the cells formed patterns, we seeded cells at 500, 1000, 2000, and 4000 cells/cm^2 in 6-well plates. The cells grew to near confluency after 3-5 days (Figure 1B) and started to exhibit the expected swirling pattern that also produced condensation and nodules after 1 month (Figure 2A)[26].

To determine if pattern-forming CVCs expressed stem cell-associated genes, we compared a CVC clone to a clone without pattern formation and osteogenic potential. We examined expression of Sox2, known to be essential to maintain self-renewal in undifferentiated embryonic stem cells, in both clones. Clones were plated in 6-well plates, and RNA was collected every 24 hours in a 5-day time course. We found that the Sox2 expression increased approximately 2-fold increase in CVCs after 1, 2, and 4 days (Figure 2B), whereas it decreased approximately 50% in non-CVCs (Figure 2B). After Day 7, CVCs developed patterns when it grew to over-confluency, whereas non-CVCs formed a monolayer of endothelial-like cells (Figure 2A). The results suggest that CVCs have a higher expression of stem cell-associated genes.

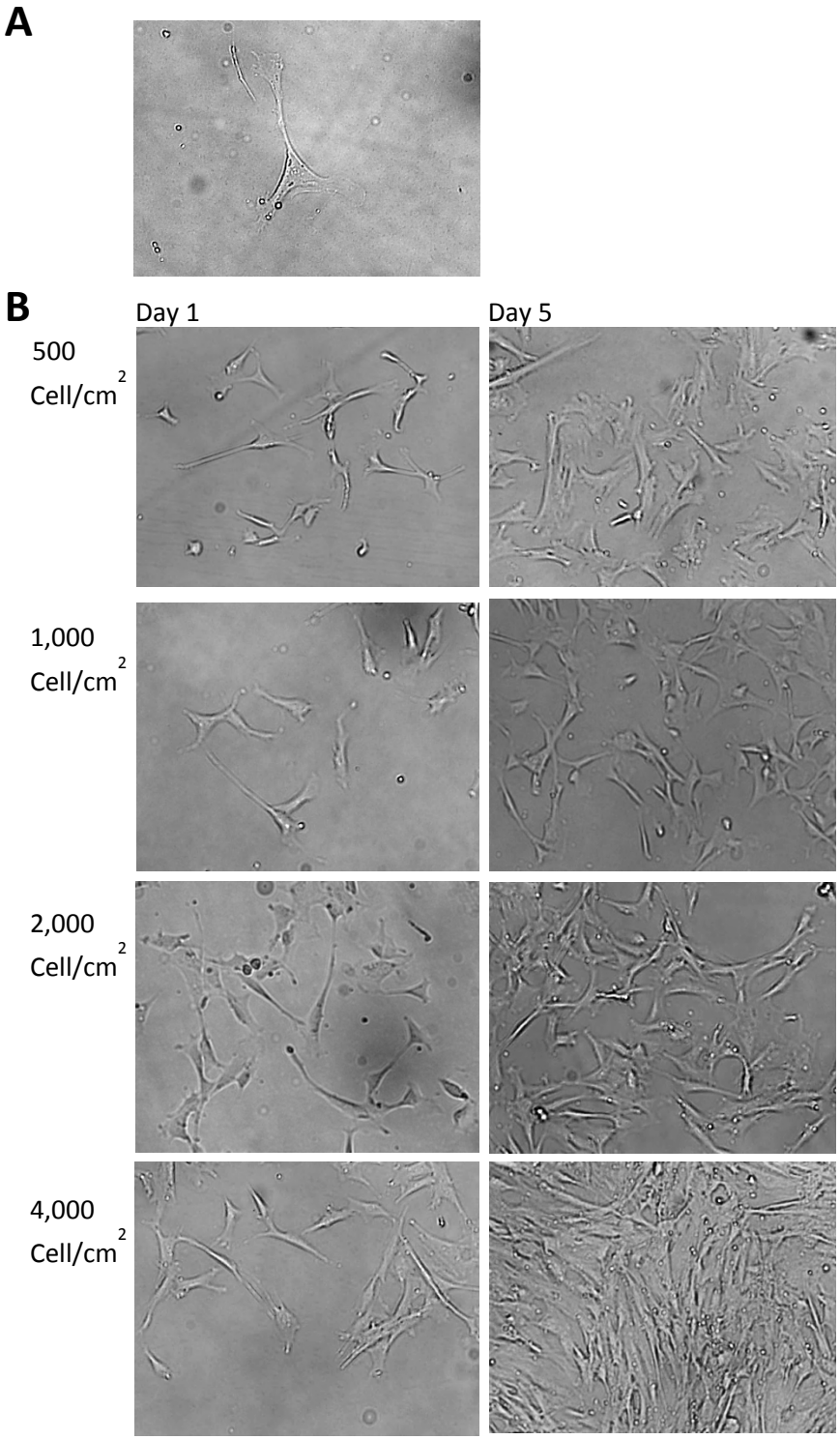


Figure 1. CVCs are cloned from BASMCs.
(A) Individual cell derived from BASMCs shows pericyte-like morphology (original magnification 10X). (B) Cloned cells were seeded at different densities (500, 1000, 2000, and 4000 cell/cm^2), and microscopic images were taken after 1 and 5 days. Swirling patterns started to form after confluency (original magnification 4X).

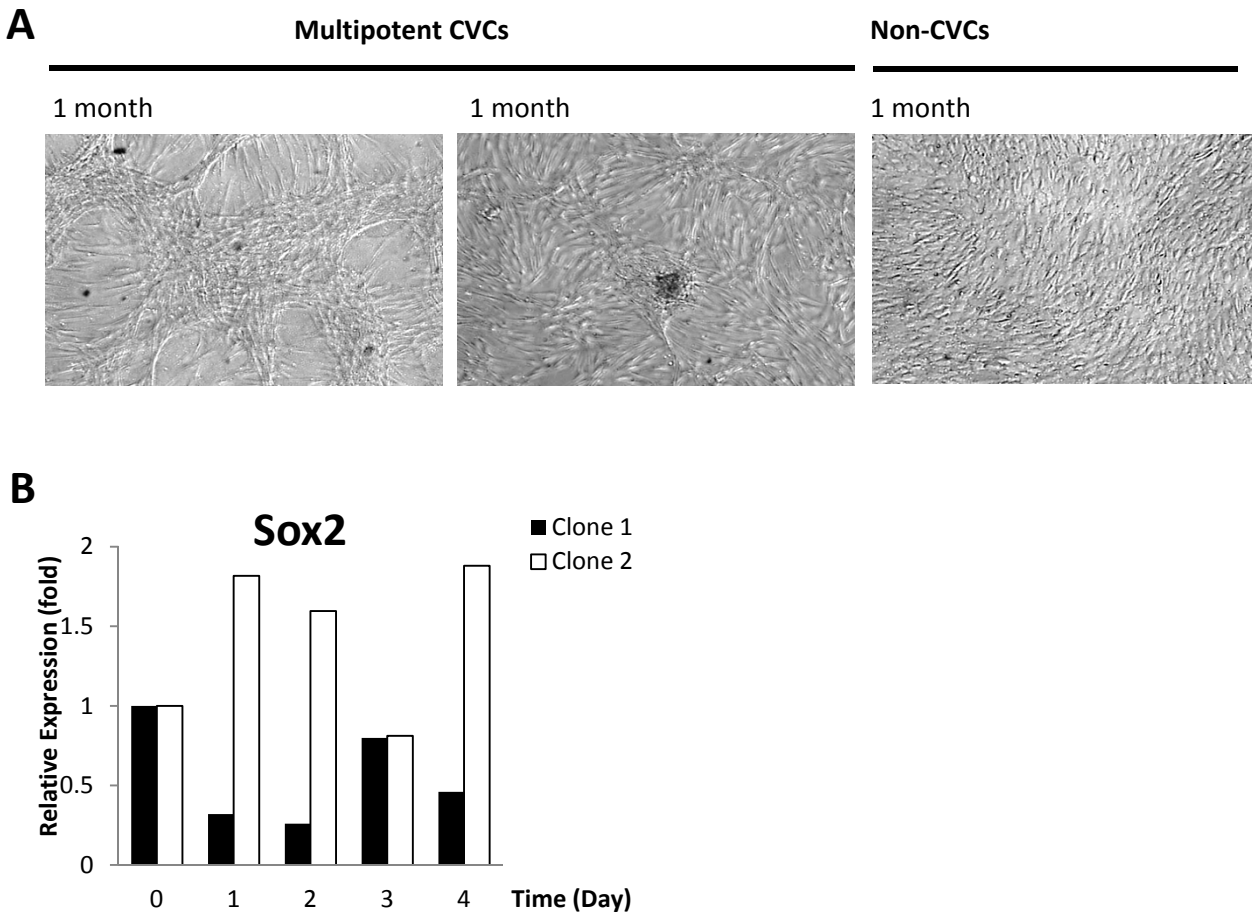


Figure 2. Sox2 expression indicates multipotentiality in CVCs.
Various CVC clones were allowed to form condensations, and microscopic images were taken after 1 month of culture. (A) CVCs with undifferentiated potential developed patterns of local alignment of cells after 1 month, and the cells eventually aggregated to form condensation and nodules, whereas non-CVCs remained in a monolayer of endothelial-like cells (original magnification 4X). (B) Sox2 expression in pattern forming CVCs during a 5-day time course was determined by real-time PCR and normalized to GAPDH.

CVC express an oscillatory pattern of stem cell-associated gene expression

We noticed that relative Sox2 expression seemed to fluctuate over the time course even though it only included five time points (Figure 2B). Because CVCs undergo stages of cell proliferation, pattern formation, and differentiation in 3-5 weeks, each stage may affect expression of stem cell markers. Therefore, we further examined stem cell-associated gene expression including Sox2, Nanog, Klf4, and Oct-3/4 in the selected CVC clone. We cultured CVCs in 12-well plates and collected RNA every 48 hours in a 2-week time course. The results suggest that expression of Sox2, Nanog, Klf4, and Oct-3/4 undergo oscillations as determined by real-time PCR (Figure 3A). The largest differences were seen in Nanog, and the least in Klf4. Both Sox2 and Nanog have increased expression in each subsequent period, whereas no clear differences are shown in Klf4 and Oct-3/4. Because Sox2 and Nanog had distinct changes of expression in a 2-week period, we focused on them in the subsequent experiments.

We also obtained microscopic images of the cells along with each RNA collection. After Day 7, CVCs developed patterns when it grew to over-confluency as expected (Figure 3B). The cells continued to develop more clear patterns and began to aggregate after 14 days as shown in previous experiments. However, there were no clear connections between the growth patterns of CVCs and the oscillations of Sox2 and Nanog expression. Further experiments are needed to determine if expression of stemness markers is a cause or an effect of CVC growth patterns. A shorter time interval between time points will be needed in such experiments in order to confirm and characterized the oscillatory expression of stemness markers.

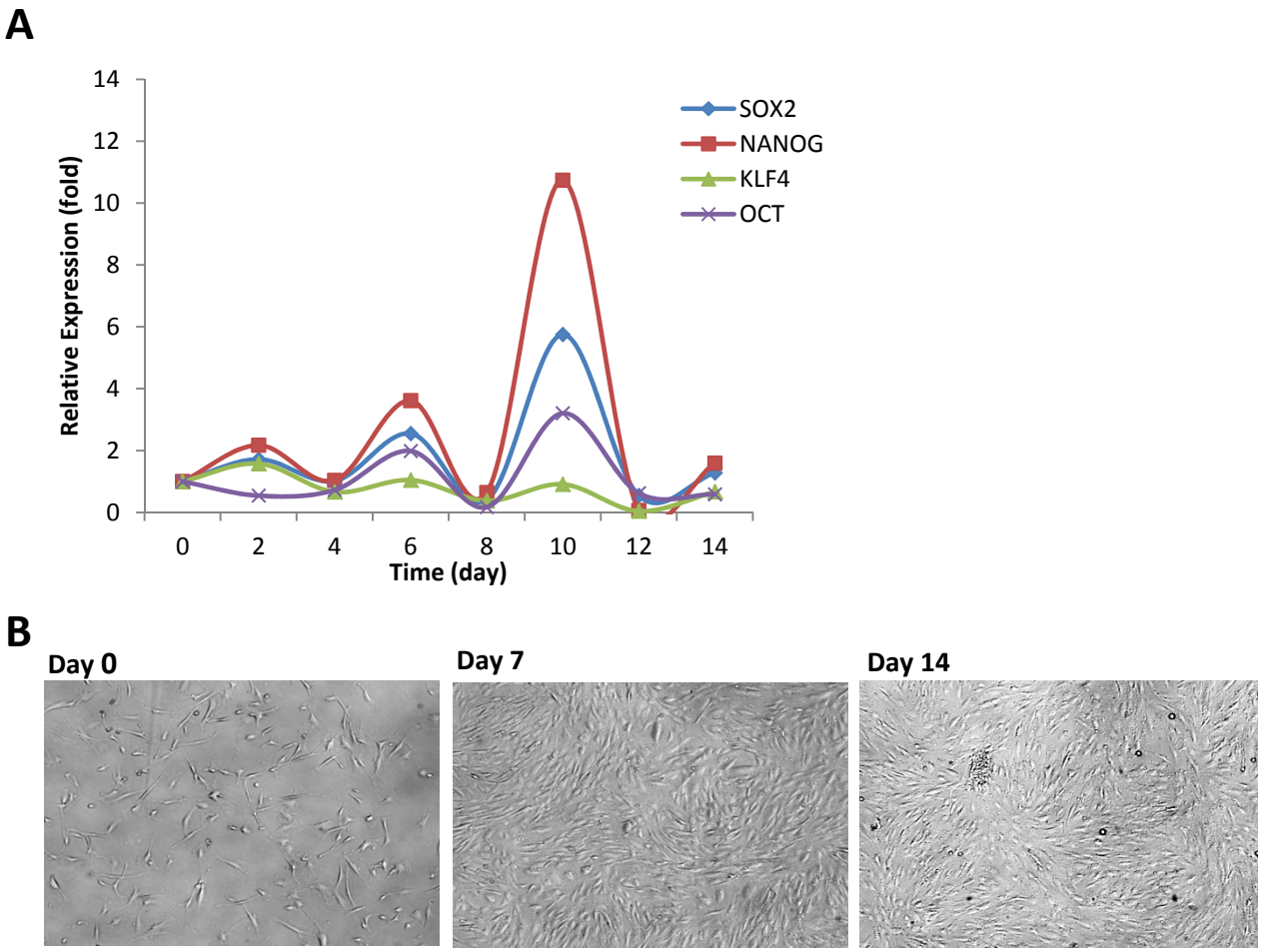


Figure 3. CVCs show oscillatory expression of Sox2, Nanog, Klf4, and Oct-3/4 during a 2-week time course.

(A) RNA was prepared every 48 hours, and expression of Sox2, Nanog, Klf4, and Oct3/4 was determined by real-time PCR and normalized to GAPDH. (B) Representative images of CVC taken after attachment (Day 0), Day 7 and Day 14. The cells reached confluency after 7 days, and began to aggregate and develop the patterns observed on Day 14 (original magnification 4X).

BMP-4 treatment affects the oscillations of Sox2 and Nanog expression

In previous studies, we showed that BMP-4 regulates expression of MGP, a BMP-inhibitor[8,29]. We also showed that BMP and MGP affect the growth pattern and size of cell condensation in CVCs[11,30]. To study the effect of BMP-4 concentration on expression of stemness markers in CVC, we cultured CVCs in 12-well plates and treated them with different BMP-4 concentrations (0, 4, or 40 ng/ml), added at the initial cell seeding. We selected 40 ng/ml of BMP-4 as one of the concentrations because it was found to be the optimal concentration to induce MGP in endothelial cells. RNA was prepared every 48 hours, and microscopic photos were obtained in a 2-week time course. The results suggested that BMP-4 (4 ng/ml) induction elevated expression of Sox2 and Nanog and may possibly havbe caused a minor shift in the expression of Sox2 expression, as determined by real-time PCR and compared to no treatment (Figure 4A&B). The oscillations of Nanog expression had higher peak expression on Day 6, 10 and 14 in CVCs treated with BMP-4 than in the controls. Furthermore, Sox2 expression was delayed by 2 days, and its peak expression was obtained on Day 4, 8, and 12. However, the results show no clear difference in Sox2 expression between the controls and the cells treated with a higher BMP-4 concentration (40 ng/ml) (Figure 4A&C). We were unable to determine Nanog expression in the experiments because of technical difficulties. The microscopic photos showed that CVCs treated with both concentrations of BMP-4 developed swirling patterns when they reached over-confluency (Figure 4D). However, the results showed no clear relations between the oscillatory expression of Sox2 and Nanog and the growth patterns. Further experiments are needed to explore the role of BMP-4 in expression of stemness markers as related to the morphology of CVCs.

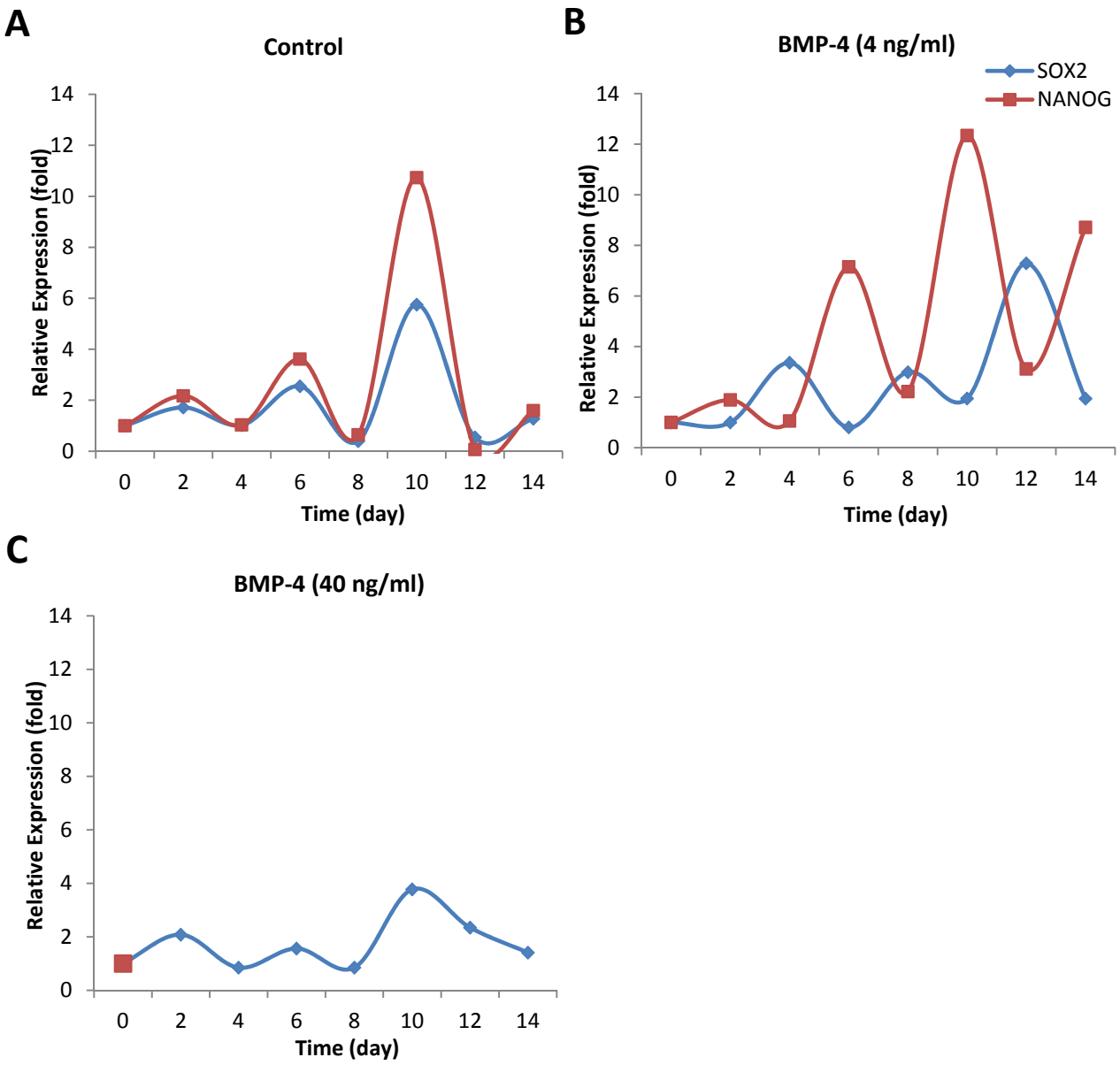


Figure 4. The effect of BMP-4 on expression of Sox2 and Nanog in CVCs.
(A) A modified Figure 3A shows the fluctuations in expression of Sox2 and Nanog in untreated CVCs during a 2-week time course. From the same CVC clone, CVCs were treated with 4 ng/ml (B) or 40 ng/ml (C) BMP-4 starting at the initial cell seeding and cultured for 14 days. RNA was prepared every 48 hours, and the expression of Sox2 and Nanog was determined by real-time PCR and normalized to GAPDH.

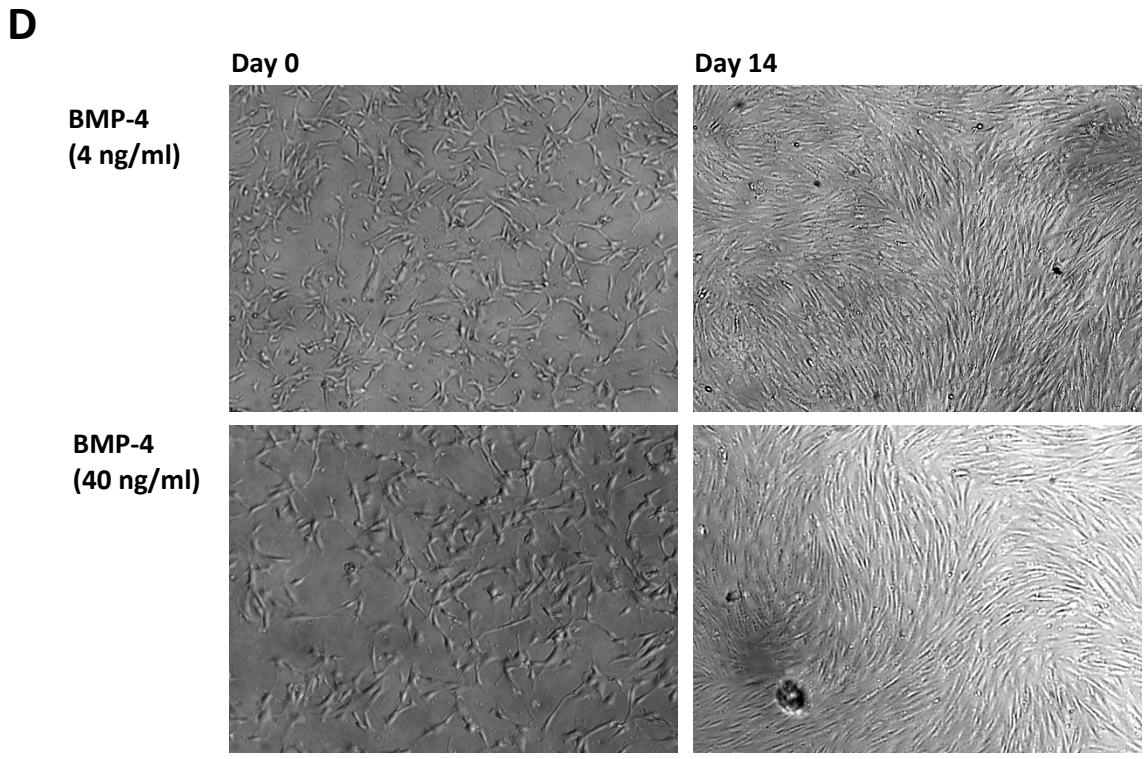


Figure 4. The effect of BMP-4 on expression of Sox2 and Nanog in CVCs (cont.).
(D) Representative images of CVCs treated with BMP-4 showed pattern formation after 14 days (original magnification 4X).

Depletion of MGP disrupts the oscillatory pattern of Sox2 and Nanog expression

To determine the effect of MGP on oscillatory expression of stemness markers, we depleted MGP using siRNA in CVCs and compared to scrambled control siRNA. We tested two MGP siRNA (siRNA1 and 2) in CVCs, and found that both siRNAs depleted MGP to <10% of the original levels even with treatment of BMP-4 (40 ng/ml), as determined by real-time PCR (Figure 5A). Because siRNA2 was a little more effective in suppressing MGP (Figure 5B), it was used in the subsequent experiments. We then compared expression of stemness markers after transfection of scrambled and MGP siRNA. The results showed that MGP depletion by siRNA increased the expression of Sox2, Nanog, and Oct-3/4 but not Klf4, suggesting that it enhanced undifferentiated potential in CVCs. The cells were subsequently treated with BMP-4 (0, 4, or 40

ng/ml) immediately after siRNA transfection. RNA was collected every 48 hours, and microscopic photos were obtained in a 2-week time course. Unexpectedly, the results showed that depletion of MGP disrupted the oscillatory expression of Sox2 and Nanog in CVCs, as determined by real-time PCR (Figure 6A). MGP depleted CVCs had lower Sox2 and Nanog expression after treatment with BMP-4 (0, 4, and 40 ng/ml) and no clear oscillations in gene expression were detected (Figure 6A, B, C&D). Overall, BMP-4 treatments had minor effects on expression of Sox2 and Nanog. Thus, the presence of MGP appeared to be essential for oscillatory expression of Sox2 and Nanog.

In our previous studies, BMP-4 was found to accelerate the growth pattern of CVCs[1,30]. Our microscopic images showed that MGP-depleted CVCs lost some of the swirling patterns when the cells reached over-confluency after 14 days (Figure 6E top). However, BMP-4 treatments may have restored some of the pattern. Mixture of vascular smooth muscle-like and endothelial-like cells were found in MGP-depleted CVCs treated with 4 ng/ml BMP-4, whereas uniformly endothelial-like cells were found in the cells without BMP treatment when they reached over-conflunency after 14 days (Figure 6E). Thus, the presence of MGP appeared to be important also for the pattern formation. However, the connections between the expression of stemness markers and the morphology of the CVCs needs further study.

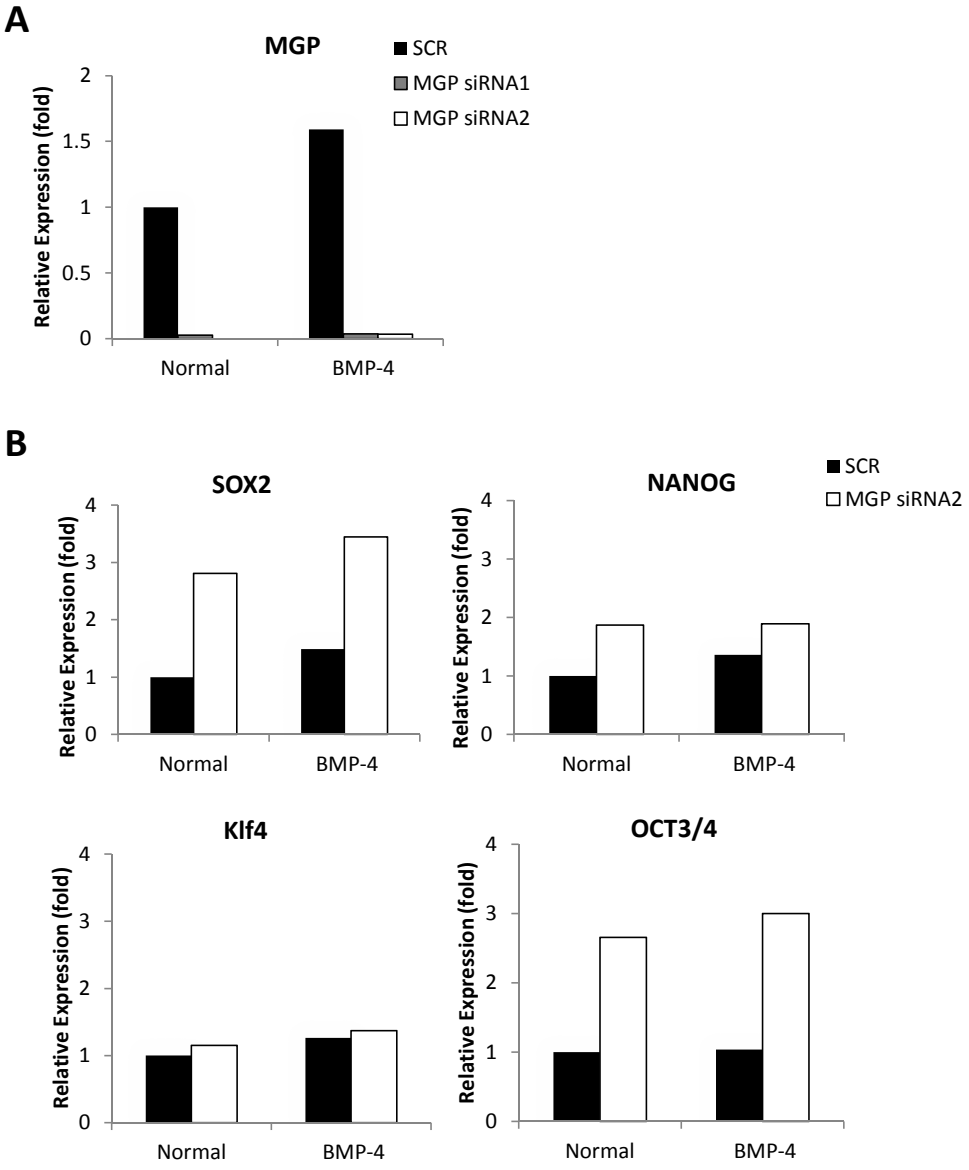


Figure 5. MGP depletion by siRNA increases expression of multipotent markers.
(A) CVCs were transfected with MGP siRNA1 and MGP siRNA2 (60 nM, 6-well plate) and cultured with or without BMP-4 (40 ng/ml). RNA was collected after 48 hours, and MGP expression was determined by real-time PCR and normalized to GAPDH. MGP siRNA2 provided the most efficient inhibition. (B) Expression of Sox2, Nanog, Klf4, and Oct-3/4 in MGP depleted CVCs was further determined by real-time PCR and normalized to GAPDH.

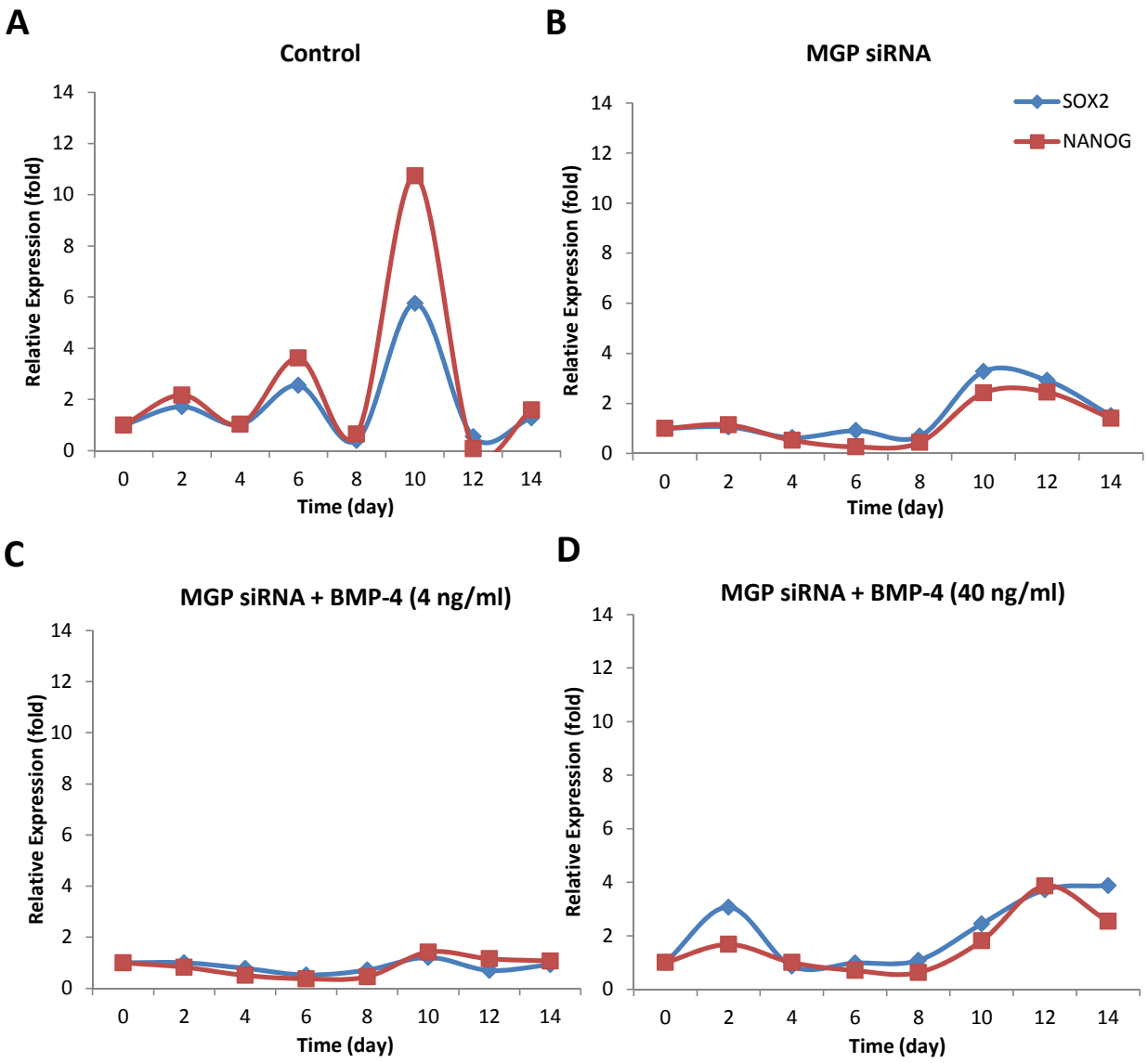


Figure 6. Depletion of MGP disrupts the oscillatory expression of Sox2 and Nanog in CVCs. (A) The graph shows the fluctuations of Sox2 and Nanog expression in control transfected CVCs during a 2-week time period. CVCs from the same clone were transfected with MGP siRNA2 (60 nM, 6-well plate) and treated with control (B), with 4 ng/ml (C) or 40 ng/ml (D) of BMP-4. RNA was prepared every 48 hours, and the expression of Sox2 and Nanog was determined by real-time PCR and normalized to GAPDH.

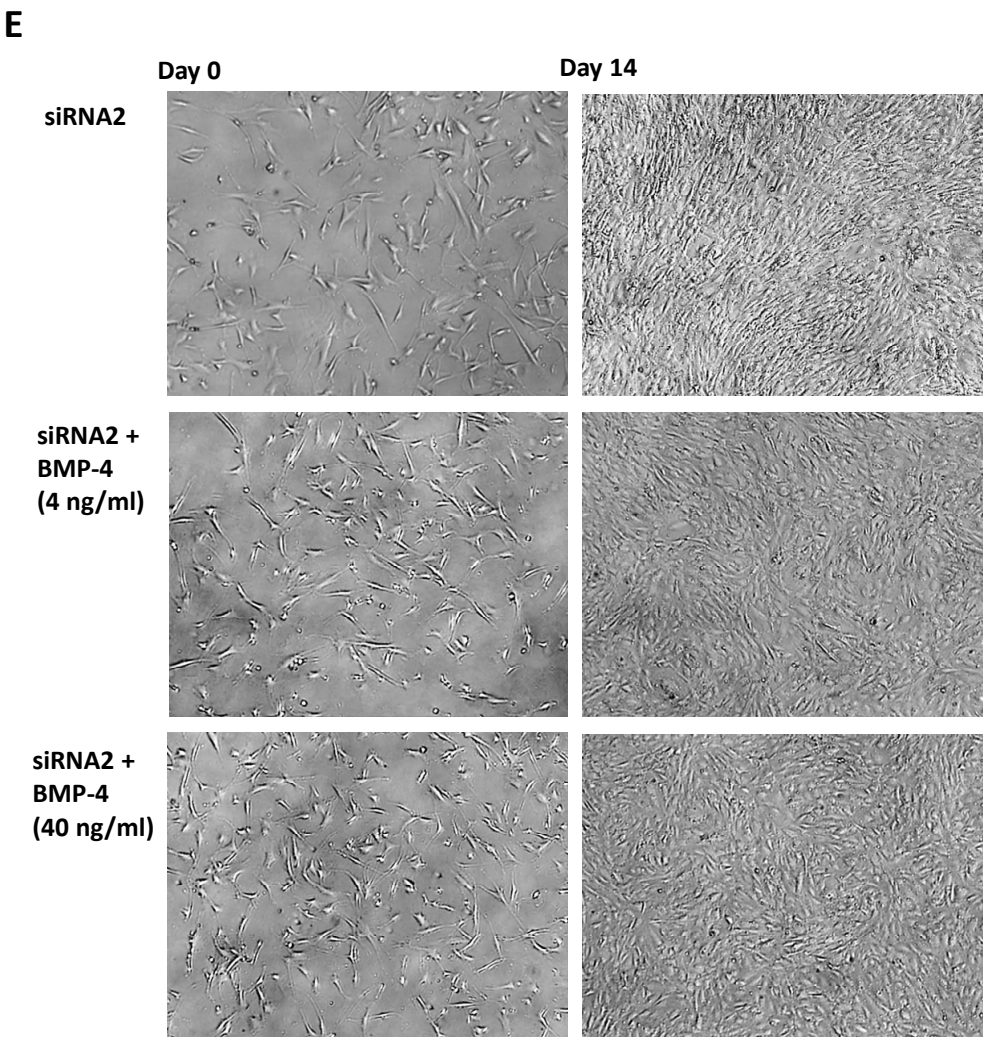


Figure 6. Depletion of MGP disrupts the oscillatory expression of Sox2 and Nanog in CVCs (cont.).
(E) Representative images show changes in morphology of the MGP depleted CVCs after 14 days. Depletion of MGP results in a more endothelial cell-like morphology (original magnification 4X).

Exploring the relations between osteogenic potential and stem cell-associated genes in CVCs

In previous studies, we[7,25,26] and others[31,32] have shown that CVCs have the ability to undergo spontaneous osteoblastic differentiation and mineralization, which is enhanced by osteogenic induction medium and BMP-4. Here we explore if osteogenic induction is more efficient when expression of stemness markers is high.

Table 1. Calcium concentration of CVC undergone mineralization after Day 10 (mg/dl)

Culture system	Calcium concentration
Control	0.052
Control with BMP-4 (40 ng/ml)	0.677
Osteogenic medium	0.696
Osteogenic medium with BMP-4 (40 ng/ml)	1.523

CVCs were treated with control medium alone or supplemented with BMP-4 (40 ng/ml) from the time of cell seeding. Because of the relatively higher expression of Sox2 and Nanog in BMP-4 treated cells on Day 10 in the previous experiments (Figure 4A), half of the dishes were placed in osteoblastic induction medium on Day 10 to see if this further promoted osteoblastic differentiation. Staining for alkaline phosphatase, an early marker of osteoblastic differentiation and determination of total cellular calcium, a late osteoblastic marker, were performed after 14 days. The results showed no clear differences in AP staining between control and BMP-4 treated samples, most likely because AP is an early marker of osteogenesis and would be induced in all cells after 14 days (Figure 7). The osteblastic induction medium enhanced osteogenesis in both control and BMP-4 treated cells (Table 1), and appeared to act synergistically with BMP-4. However, further experiments are needed to determine if it makes a difference whether the induction medium is added when Sox2 and Nanog expression is high or low.

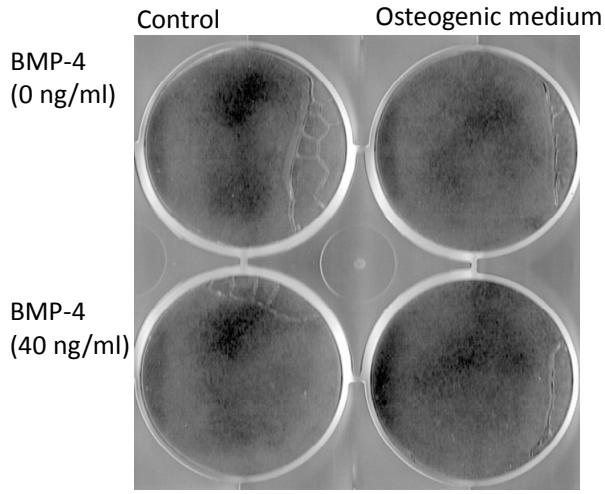


Figure 7. Alkaline phosphatase staining of CVCs treated with BMP-4l and osteogenic medium. CVCs were cultured in a 6-well plate and treated with BMP-4 (0 or 40 ng/ml) staining at the initial cell seeding. The culture medium was replaced with osteoblastic induction medium in half of the wells on Day 10. AP staining was performed on Day 14.

DISCUSSION

In this study, we showed that expression of stem cell-associated genes oscillated in selected clones of multipotent CVCs as they progressively developed swirling patterns after confluency. The largest difference in amplitude in the oscillations was found in Sox2 and Nanog expression. Furthermore, our results showed that the effects of BMP-4 and MGP on the oscillations and the pattern formation in CVCs differed. BMP-4 had minor effects on the gene expression, whereas the depletion of MGP disrupted the oscillations and pattern formation. Overall, our results suggested the existence of oscillatory patterns of so-called stemness markers in CVCs, a novel finding in vascular cells, and that BMP inhibition by MGP was essential for the regulation of gene expression and morphology in CVCs.

Biological oscillators, systems that generate a periodic variation in the state of a cell, tissue or organism, were first studied in vertebrate segmentation. The segmental pattern of the spine is established early in development when segments of vertebrates, the somites, are rhythmically and sequentially produced from the presomitic mesoderm, triggered by the Notch, Wnt/β-catenin, and fibroblast growth factor pathways in an oscillating genetic network known as the segmentation clock[6,33-35]. In vascular development and calcification, patterns and repeated morphogenetic processes have been observed in CVCs and may be important. The cells aggregate into patterns in vitro, which may correspond to calcified structure within the artery wall[26,36], but the exact patterning mechanism is still not known. Previously, Garfinkel et al. investigated the effect of BMP and MGP on the pattern formation in CVCs, and found that it could in large part be predicted with mathematical modeling[11]. Because studies have reported that several markers of stemness show patterned response in the other cells, we hypothesized that

multipotent CVCs show periodic changes in gene expression similarly to known oscillatory systems, and that there is a relationship between gene expression and patterns in CVCs.

Multipotent CVCs have been shown to have self-renewal capacity and ability of spontaneously forming patterned condensations[1,7]. To our knowledge, however, oscillations in stem cell-associated or other genes in CVCs have not been reported. Other studies have reported that Sox2 and Oct-3/4 are crucial in Nanog regulation and self-renewal in VMCs[37]. We found that Sox2 and Nanog expression was elevated when morphogenetic changes occurred in CVCs. The cells seemed to experience approximately a wave of three cycles of fluctuations, and the highest oscillation was found when swirling patterns were formed, suggesting that gene oscillations and pattern formation may be connected in CVCs.

Out study is limited by several factors. First, the limited number of time points; it is still not clear whether the oscillations trigger or respond to changes in morphology. Further studies are needed to better define the oscillations with more time points in the time course. Second, choosing suitable CVC clones with enhanced ability of forming patterns and nodules would also strengthen our studies of the oscillatory model. We have previously reported on fast nodule forming CVCs[26], in which it would be easier to connect oscillations to pattern formation. Third, it will be necessary to better characterize oscillations in regards to frequency, periodicity, and potential synchronization of oscillations of different genes[35]. They will also need to be correlated to repetition in cellular patterns. It is still not known if the difference among single-cell, local, and overall oscillators and their connection to CVC morphology. Although we believe that oscillations are important in vascular morphology, understanding the mechanisms will be a challenge.

Another important aspect of oscillations is the potential disruption by changes in local conditions. BMPs are known morphogenic factors with the ability to stimulate vasculogenesis in the embryo[12]. However, BMP-4 had minor effects on the stem cell-associated gene expression in our experiements. It did appear, however, that a small dosage of BMP-4 (4 nb/ml) seemed to enhance Sox2 and Nanog expression, whereas a large dosage of BMP-4 (40 ng/ml) seemed to suppress the oscillations at the end of the time course. This might point to a BMP-4 dosage dependent effect on oscillatory gene expression, which could be important when BMPs participate in morphogenic gradients during embryogenesis[8,27]. It would be important to continue examining the effect of different BMP-4 concentration in CVCs and its relation to cellular patterns.

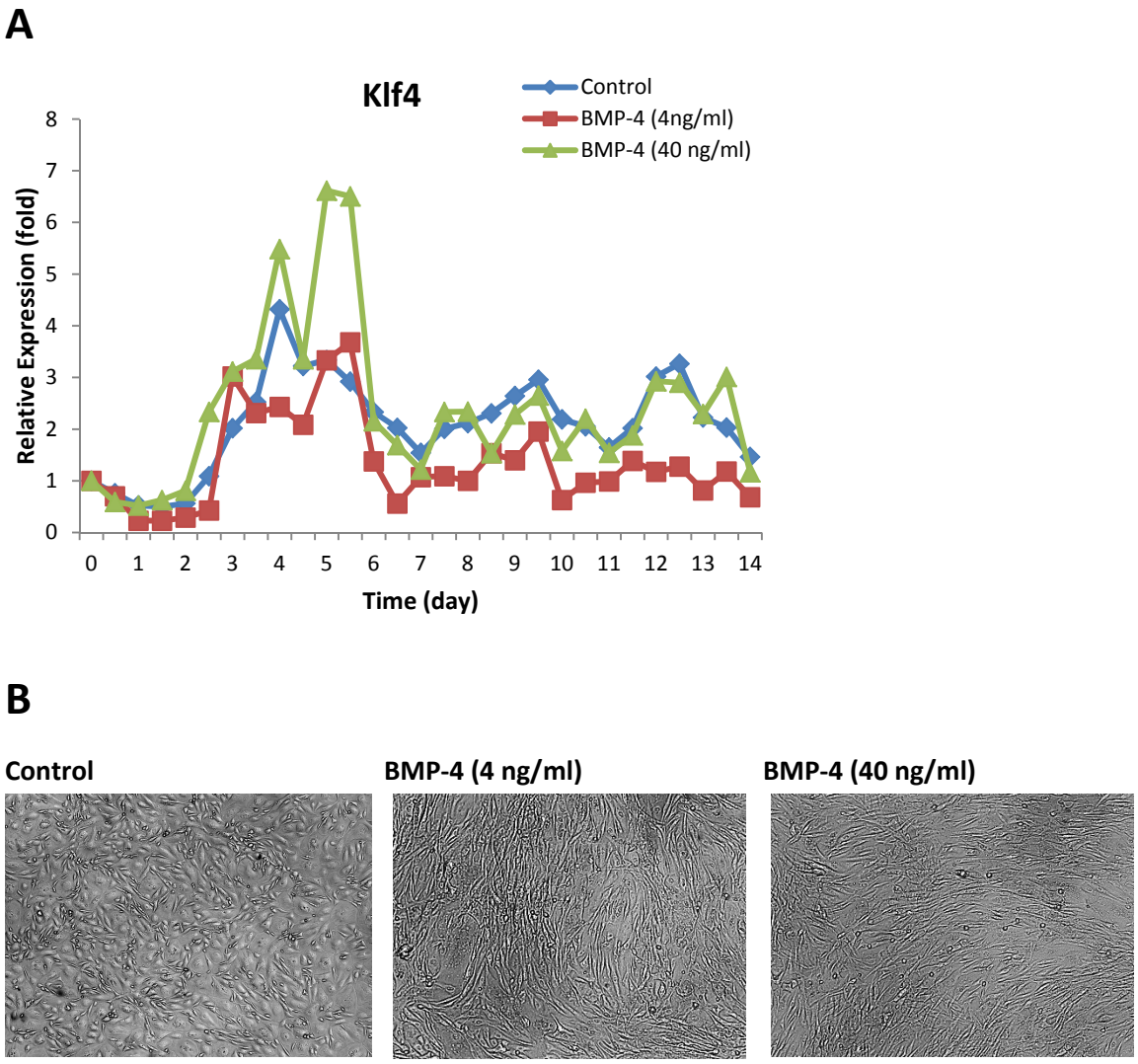
MGP has been previously shown as a BMP-4 inhibitor and affects pattern formation in CVCs[8,11,28]. Our study showed that the depletion of MGP resulted in the disruption and suppression in Sox2 and Nanog expression. CVCs eventually differentiated, but the swirling patterns were not observed after 2 weeks. The results suggested that the presence of MGP, an inhibitor of BMP-4, may be more important to maintain oscillations than the exact concentration of BMP-4. Further experiments are needed to examine the importance of MGP for oscillations.

We have previously shown that activin-like kinase receptor 1 (ALK1) is an important link between MGP, where ALK1 mediates BMP-4 induction of MGP, thereby establishing a balance between BMP-4 and MGP[8,17]. Further studies on the expression and the activity of ALK1 may provide an explanation of the loss of stem cell-associated oscillation and pattern formation and the presence of non-CVCs in the culture.

Different patterns are formed in different CVC clones, and we have continued to include more clones in our studies. Using a different CVC clone, we performed a 2-week time course

and collect RNA every 12 hours. Analysis of this time course showed that Klf4, rather than Sox2 and Nanog, was expressed in oscillatory pattern with approximately three periods (Appendix Figure I). Interestingly, BMP-4 treated CVCs expressed only one period of Klf4 oscillation before the expression was suppressed 7 days into the time course, suggesting that BMP-4 disrupted the oscillations in this case. Clearly, further experiments are needed to develop a model of oscillating genetic network in CVCs.

In summary, our study suggests the presence of oscillations in stem cell-associated gene expression in pattern forming CVCs. The largest amplitudes in the expression were detected for Sox2 and Nanog. BMP-4 and MGP appeared to affect the characteristic of the oscillations, suggesting that there may be a connection between the properties of the oscillations and morphogenetic processes in CVCs. In recent years, vertebrate segmentation has sparked interest in oscillatory regulation of signaling as a mean to cellular response. Additionally, studies have reported that patterned genetic response in other cells. Our results suggest that CVCs may also have such oscillatory genetic network, and that understanding this network may reveal its connection to cellular pattern formation and ultimately to patterns of disease.



Appendix Figure 1. BMP-4 may alter oscillations in Klf4 expression in a different CVC clone. (A) RNA was prepared every 12 hours, and expression of Klf4 was determined by real-time PCR and normalized to GAPDH. (B) Representative images of CVC taken after Day 14 (original magnification 4X).

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