The combination of BMP12 and KY02111 enhances tendon differentiation in bone marrow-derived equine mesenchymal stromal cells (BM-eMSCs)

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The Wingless and Int-1 (WNT) and bone morphogenic protein/growth differentiation factor (BMP/GDF) signalling pathways contribute significantly to the development of the musculoskeletal system. The mechanism by which they contribute is as follows: BMP/GDF signalling usually promotes tendon differentiation, whereas WNT signalling inhibits it. We hypothesised that inhibiting WNT and subsequently stimulating BMP signalling may enhance the tenogenic differentiation of stem cells. The objective of this study was to determine whether a combination of WNT inhibitor (KY02111) and BMP12/GDF7 protein could enhance the differentiation of bone marrow-derived equine mesenchymal stromal cells (BM-eMSCs) into tenocytes. Cells were cultured in five treatments: control, BMP12, and three different combinations of BMP12 and KY02111. The results indicated that a 1-day treatment with KY02111 followed by a 13-day treatment with BMP12 resulted in the highest tenogenic differentiation score in this experiment. The effect of KY02111 is dependent on the incubation time, with 1 day being better than 3 or 5 days. This combination increased tenogenic gene marker expression, including SCX, TNMD, DCN, and TNC, as well as COL1 protein expression. In conclusion, we propose that a combination of BMP12 and KY02111 can enhance the in vitro tenogenic differentiation of BM-eMSCs more than BMP12 alone. The findings of this study might be useful for improving tendon differentiation protocols for stem cell transplantation and application to tendon regeneration.
Key words: *BMP12, horse, KY02111, tendon*

Introduction

Tendinitis as a result of an injury is one of the most common health issues in sport horses [39]. Even after conventional treatment (NSAIDs, physical therapy, and an exercise programme), tendon fibrosis persists, and the recovered tendon is weaker and more susceptible to re-injury due to increased numbers of abnormal collagen types and fibroblasts and a concurrent decrease in tenocyte number [2, 30].

Nowadays, equine mesenchymal stromal cells (eMSCs) are used to treat various equine diseases, including arthritis and tendinitis [12, 18, 28, 34]. The therapeutic effect of MSCs on the tendon is believed to be due to paracrine factors that modulate inflammation and promote cell differentiation, rather than to direct differentiation of MSCs [9, 13, 32]. Tendinitis treatment with eMSCs demonstrated a clinical outcome superior to conventional therapy in horses, as well as improved collagen organisation and decreased fibrosis [1, 12, 28].

While it is common to transplant undifferentiated MSCs, recent studies have demonstrated that targeted partial differentiation results in superior *in vitro* and clinical outcomes [6, 8, 29, 40]. Additionally, tenogenically induced MSCs have been shown to decrease the risk of ectopic bone formation following transplantation [19]. Tenogenic differentiation of equine mesenchymal stem cells *in vitro* is frequently accomplished by supplementing the culture medium with growth and differentiation factors such as bone morphogenic protein/growth differentiation factor (BMP/GDF) and TGF family proteins [18, 32]. However, no standard protocol for tendon differentiation exists, and the tenocyte differentiation efficiency is still limited [22].

In vivo embryonic development has been used to gain a better understanding of mesoderm differentiation and to develop protocols for *in vitro* differentiation [36]. During embryonic development, osteochondroprogenitor cells are formed, and these cells can differentiate into osteogenic (bone) or tenochondrogenic progenitor cells. Tenochondrogenic progenitor cells can subsequently differentiate into progenitors of chondrocytes (cartilage) or tenocytes (tendon) [3, 7]. Notably, mesoderm differentiation is regulated by signalling pathways, including the Wingless and Int-1 (WNT)/β-catenin, Notch, and BMP/GDF signalling pathways [5, 36]. The WNT/β-catenin signalling pathway has been shown to inhibit tenogenesis [17] and chondrogenesis [14, 17] but to promote osteogenesis [16, 21]. Conversely, BMP/GDF signalling promotes tenogenic differentiation [15, 27].

Our hypothesis was that increasing the tenochondrogenic progenitor and then stimulating the tenocyte lineage would enhance eMSC tenogenic differentiation. Inhibition of WNT signalling may be used to increase tenochondrogenic progenitors, whereas stimulation of the BMP/GDF signalling pathway may be used to promote tenogenic differentiation. The objective of this study was to determine whether the combination of a WNT inhibitor and BMP/GDF stimulator could enhance the differentiation of bone marrow-derived equine mesenchymal stromal cells (BM-eMSCs) into tendon. In this experiment, we used KY02111 as the WNT/β-catenin signalling pathway inhibitor [24] and BMP12 (also known as GDF7) as the BMP/GDF signalling pathway stimulator [32]. BMP12 has been shown in the majority of previous studies to promote tenogenic differentiation [15, 27].

Materials and Methods

This research project was approved by the Animal Care and Use Committee of the Faculty of Mahidol University.

eMSC culture

BM-eMSCs in this experiment were obtained from a stem cell bank at Mahidol University. These cells have already been characterised and transplanted by our research team [18, 42]. Flow cytometry showed that they expressed for CD29 (90%), CD90 (90%), CD14 (14%), CD34 (2%), CD44 (4%), CD45 (0.5%), CD73 (11%), and CD105 (3%). PCR revealed that they are positive for CD90, POU5F1, CD14, and CD44 but negative for CD105, NANOG, SOX2, CD79a, and DRB. They were capable of differentiating into osteocytes, chondrocytes, adipocytes, and tenocytes [18]. This study was done in a period similar to the above two previous reports [18, 42].

Frozen eMSCs from passage 3 were used. Cell culture was performed in accordance with a previously published protocol [18, 42]. Briefly, cells were thawed at 37°C and then washed in a 15 ml tube with 5 ml culture medium (Minimum Essential Medium plus 10% foetal bovine serum; FBS). They were subsequently centrifuged at $1,500 \times g$ for 5 min, and the supernatant was then removed. The pellet was resuspended in 5 ml of culture medium and then transferred to a T25 flask and cultured in a humidified incubator with 5% CO_2 at 37°C. The three MSCs lines used in this study were isolated from three thoroughbred horses. Each cell line was tested in triplicate.

Strategy for tenogenic differentiation

A summary of the strategy is shown in Fig. 1. First, dexamethasone, ascorbic acid, and glucose were added to the differentiation medium to promote osteochondrogenic differentiation [4, 10, 35, 45], followed by the addition of KY02111, the WNT inhibitor, to inhibit osteogenic differentiation but induce tenochondrogenic differentiation. Finally, BMP12 was introduced to promote tenogenic differentiation [15, 27].

The differentiation medium comprised Dulbecco's Modified Eagle Medium with high glucose (Thermo Fisher Scientific, Waltham, MA, U.S.A.), 10% FBS (Sigma-Aldrich, St. Louis, MO, U.S.A.), 0.1 μM dexamethasone (Sigma-Aldrich), 50 μg/ml ascorbic acid (Sigma-Aldrich), and 1% antibiotic-antimycotic solution (Thermo Fisher Scientific). Additionally, 10 μM KY02111 (Selleckchem, Houston, TX, U.S.A.) and 200 ng/ml BMP12 (PeproTech, Cranbury, NJ, U.S.A.) were added at various time points during the experiment. eMSCs were seeded in 35 mm culture dishes at a density of 3×10^4 of cells and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. The medium was replaced with fresh medium every 3 days.

Experimental design

The eMSCs were divided into the following five groups (Fig. 2): 1) control group, in which cells were cultured in

differentiation medium without BMP12 and KY02111; 2) BMP group, in which cells were cultured in differentiation medium containing BMP12; 3) BMPKY1 group, in which cells were cultured in differentiation medium containing KY02111 for 1 day and BMP12 for 14 days; 4) BMPKY3 group, in which cells were cultured in differentiation medium containing KY02111 for 3 days and BMP12 for 12 days; and 5) BMPKY5 group, in which cells were cultured in differentiation medium containing KY02111 for 5 days and BMP12 for 10 days. On day 15 (the end of cell culture), cells were harvested for gene and protein expression analysis.

Analysis of tenogenic markers

On days 14 to 15, BM-eMSCs were collected for the purpose of determining particular tenogenic markers. Gene expression of Tenascin-C (TNC), Decorin (DCN), Scler-axis (SCX), and Tenomodulin (TNMD) was detected and quantified using quantitative polymerase chain reaction (qPCR), while type I collagen (COL1) protein expression was observed and quantified using immunofluorescence microscopy.

RNA extraction and cDNA synthesis

To study gene expression, total RNA was extracted from eMSCs using a column-based nucleic acid extraction kit (Quick-RNA^{TM}, Zymo Research, Irvine, CA, U.S.A.). After extraction, the quantity and quality of RNA were determined with a Nanodrop spectrophotometer (Thermo Fisher Scientific). RNA was converted to cDNA using a SuperScript$^®$ VILO^{TM} cDNA Synthesis Kit, according to the manufacturer's instructions (Thermo Fisher Scientific).

qPCR

The qPCR was performed according to our previous studies [38, 42]. Briefly, qPCR was carried out on a Rotor-Gene Q instrument (Qiagen, Hilden, Germany). The PCR buffer was KAPA SYBR$^®$ Fast qPCR Master Mix (Kapa Biosystems, Wilmington, MA, U.S.A.). The following qPCR reaction protocol was used: Step 1 was pre-denaturation at 95°C for 1 min. Step 2 was cycling at 95°C for 5 sec (denaturation) and 60°C for 20 sec (annealing); this step was performed for 35 cycles. Step 3 was a final extension at 72°C for 3 min. Step 4 was post-PCR melting, in which the temperature was increased from 72°C to 99°C in 1-degree increments with a hold for 15 sec on the first step and for 4 sec on each subsequent step. The Rotor-Gene Q software was used to analyse the melting curve and measure comparative gene expression. Comparative gene expression was normalised with a reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and set relative
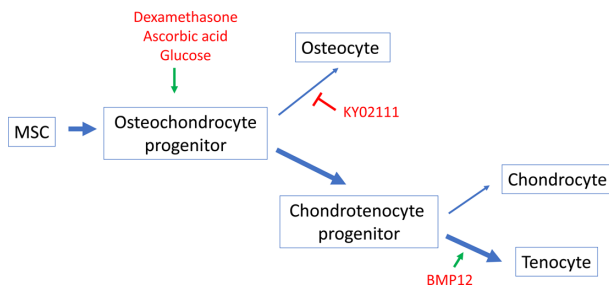

Fig. 1. Strategy for tenogenic differentiation of bone marrow-derived equine mesenchymal stromal cells (BM-eMSCs). The thickness of the blue arrows represents the strength of differentiation. The green arrows indicate that the chemicals (red text) added to the medium can stimulate differentiation, while the red line represents inhibition.
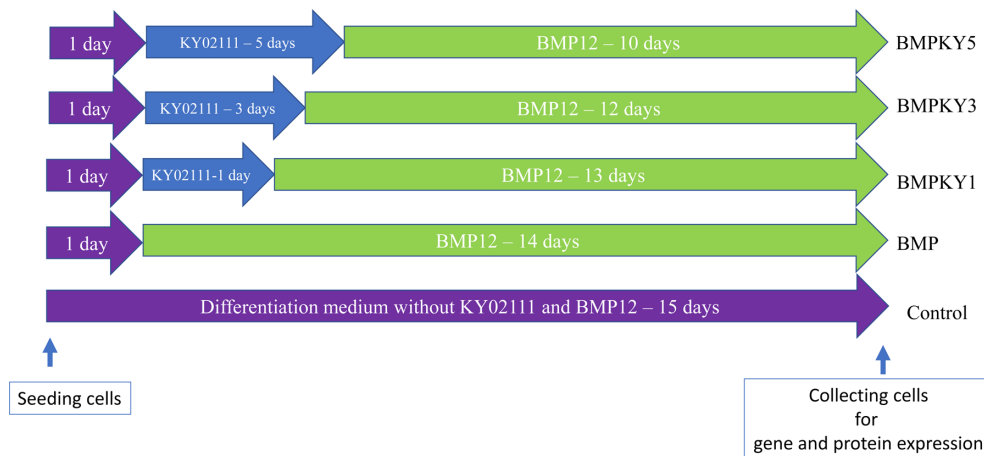

Fig. 2. *In vitro* tenogenic differentiation experiments for equine mesenchymal stromal cells (eMSCs) under different conditions with KY02111 (Wingless and Int-1 (WNT) inhibitor) and BMP12 (Growth factor) before gene expression and immunofluorescence studies.

Table 1.   Primers sequences used for qPCR

Gene	Forward primer sequence (5'>3')	Reverse primer sequence (5'>3')	Tm (°C)	Product size (bp)
GAPDH	CACTGAGGACCAGGTTGTCT	GGGTCAAGTTGGGACAAGCA	60	262
TNC	CGGAAACCAGACATCCACCA	AGGTGCAGGTAAGTAAGTGGC	60	133
DCN	AGGGCTCCTGTGGCAAATC	GGCACTTTGTCCAGACCCAA	60	295
SCX	CCAGCTACATCTCGCACCTG	GCGGTCCTTGCTCAACTTTC	60	221
TNMD	GGCGGGTTATCTGTCGTG	TACCAGGAGCCAAATGCC	60	258

to the control group. The PCR reaction was performed in triplicate. Primer sequences are shown in Table 1.

Immunofluorescence assays

After 14 days of differentiation (Fig. 2), cells in each group were sub-cultured at an 80% confluence density in a chamber slide (Nalgene, Rochester, NY, U.S.A.), and culture was continued for 24 hr. Cells were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS) for 10 min at room temperature and then washed twice with PBS for 5 min. Next, they were permeabilised with 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 5 min and washed with PBS for 5 min. To block non-specific binding, cells were incubated with 3% bovine serum albumin (BSA; Sigma-Aldrich) in PBS at room temperature for an hour. After that, they were incubated overnight at room temperature with a primary antibody, mouse anti-collagen type I (1:500) antibodies (Millipore, CA, U.S.A.), in 1.5% BSA and then washed twice with PBS for 5 min. The cells were then incubated with a secondary antibody conjugated with Alexa Fluor 488 (goat anti-mouse; Abcam, Cambridge, U.K.) for 1 hr at 4°C in the dark. Following that, the unbound antibodies were removed by washing twice with 0.03% Triton X-100 in PBS at room temperature for 5 min. A coverslip was mounted with ProLong Gold Antifade Mountant with DAPI medium (Thermo Fisher Scientific). The stained cells were imaged and counted using a model BX51 fluorescence microscope (Olympus, Tokyo, Japan). Cells in three visual fields were counted and calculated as the percentage of positive (collagen type I) cells.

Differentiation score

In order to compare the treatments, we developed the following differentiation scoring system: the highest expression received a score of 3 (statistically different from expression scored as 2), medium expression received a score of 2 (statistically different from expression scored as 1), lowest expression received a score of 1, and no expression received a score of 0.

Statistical analysis

Mean ± standard error (SE) values were calculated for gene expression (TNC, DCN, SCX, and TNMD). Statisti-

cally significant differences in gene expression among the experimental groups were determined using repeated measures ANOVA followed by multiple comparisons using the Tukey post hoc test. Protein expression (COL1) is expressed as the percentage of positive cells, and the statistical significance of differences was calculated using the chi-square test. Differences with a P-value of <0.05 were considered statistically significant. The statistical analysis was performed using jamovi version 1.8.1, which is a publicly downloadable tool (https://www.jamovi.org).

Results

Gene and protein expression levels of tenogenic markers are shown in Table 2. Differentiation of eMSCs with in the BMPKY5 group (incubated with KY02111 for 5 days) resulted in a significant increase (P<0.05) in TNC and DCN gene expression compared with the other groups. SCX and TNMD gene expression was significantly higher in the BMPKY1 group (incubated with KY02111 for 1 day) than in the other groups (P<0.05).

For COL1 staining (Fig. 3), the number of positive cells was significantly higher (P<0.05) in the treatments that contained KY02111 and BMP12 (BMPKY1, BMPKY3, and BMPKY5) than in the BMP and control groups.

The findings indicate that BMP12 or a combination of BMP12 and KY02111 promotes tendon differentiation more effectively than the control culture. The score for tendon differentiation was shown in Table 3, and the best score was obtained when the BMPKY1 treatment was used (KY02111 treatment for 1 day followed by 14 days of BMP12 treatment).

Discussion

We demonstrated in this study that a combination of KY02111 and BMP12 could enhance the tenogenic differentiation of eMSCs more than BMP12 alone. Supplementation with the WNT inhibitor, KY02111, for 1 day and then BMP12 for 14 days resulted in the highest tenogenic differentiation score in this experiment.

Other WNT inhibitors, such as SM04755 and IWR1, have been shown to induce tenogenic differentiation in a

Table 2. Comparison of gene expression and percentages of positive cells for tenogenic markers among treatments

Group	Comparative gene expression (Mean ± SE)				Percentage of positive cells
	TNMD	SCX	DCN	TNC	COL1
Control	1.00 ± 0.00^a	1.00 ± 0.00^a	1.00 ± 0.00^a	1.00 ± 0.00^a	$0.00\%\ (0/153)^a$
BMP	11.58 ± 4.34^b	1.36 ± 0.21^a	1.53 ± 0.12^a	1.43 ± 0.17^a	$50.32\%\ (78/155)^b$
BMPKY1	52.95 ± 19.38^c	2.16 ± 0.36^b	1.60 ± 0.12^a	1.58 ± 0.15^a	$53.72\%\ (65/121)^{bc}$
BMPKY3	1.81 ± 0.63^a	0.98 ± 0.05^a	1.36 ± 0.31^a	1.26 ± 0.19^a	$65.65\%\ (86/131)^c$
BMPKY5	1.55 ± 0.04^a	0.86 ± 0.07^a	3.32 ± 0.51^b	3.27 ± 0.37^b	$55.13\%\ (86/156)^{bc}$

Average gene expression was calculated for the three cell lines and repeated in triplicate. [a–c]Significant difference ($P<0.05$). TNMD: Tenomodulin; SCX: Scleraxis; DCN: Decorin; TNC: Tenascin-C; COL1: type I collagen.
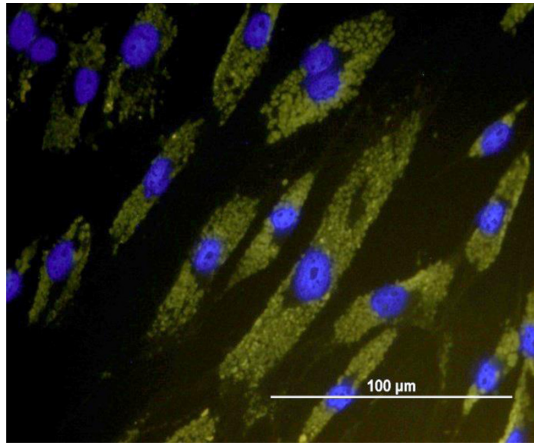

Fig. 3. Immunofluorescence image of tenogenically differentiated eMSCs. Cells were positively stained with a primary antibody specific to type I collagen (COL1). Positive staining was visualized with a secondary body conjugated with Alexa Fluor 488 (green colour in the cytoplasm), while nuclei were stained with DAPI (blue).

Table 3. Comparison of differentiation scores for tenogenic marker expression among treatments

	TNMD	SCX	DCN	TNC	COL1	Total
Control	1	2	2	2	0	9
BMP	2	2	2	2	2	10
BMPKY1	3	3	2	2	3	13
BMPKY3	1	2	2	2	3	8
BMPKY5	1	2	3	3	3	12

The highest expression received 3 points, medium expression received 2 points, the lowest expression received 1 point, and no expression received 0 points. TNMD: Tenomodulin; SCX: Scleraxis; DCN: Decorin; TNC: Tenascin-C; COL1: type I collagen.

manner similar to KY02111. SM04755 has been shown to increase the expression of tenogenic markers, such as SCX, TNMD, TNC, Mohawk (MKX), thrombospondin 4 (THBS4), and type I and type III collagens [11], while IWR1 has been shown to increase the expression MKX, TNMD, and SCX [17]. Conversely, WNT stimulators such as BIO have been reported to reduce the expression of SCX, MKX, and TNMD in monolayer cultures of rat tendon cells, eMSCs, and human MSCs [17, 25]. The current study also demonstrated that the incubation time for the WNT inhibitor is critical because 1 day of incubation seemed superior to 3 or 5 days. This result could be explained by a previous study that reported that inhibiting the WNT signalling pathway for an extended period results in the apoptosis of rat tendon stem cells [41].

Apart from WNT pathway molecules, the roles of other small molecules in tendon differentiation have been investigated. For example, a combination of Oxo-M and 4-PPBP (FAK and ERK1/2 signalling pathway) synergistically increases the expressions of tenogenic markers (Col-I, Col III, Tn-C, Vim, Tnmd, Scx) in tendon stem/ progenitor cells (TSCs) [37]. Thus, not only is it essential to use small molecules to improve tendon differentiation for stem cell transplantation, but it also might be possible to use certain molecules as drugs to promote tendon regeneration following injury [11].

The present study and other previous studies found that BMPs play a crucial role in tendon development and regeneration. For instance, our study and others have demonstrated that BMP12 can promote tenogenic differentiation [15, 27] by stimulating SCX expression [20] via the Smad1/5/8 pathway [31]. Overexpression of BMP12 can promote tendon regeneration and formation [23]. Other BMP members, such as BMP-2 and BMP-7, also play a critical role in tendon healing via Smad pathways [44]. Among tenogenic markers, a transcription factor SCX is considered an early marker for tendon differentiation. SCX can directly induce the expression of other makers, such as TNMD, COL1, and DCN [33, 43], as well as indirectly stimulate TNC expression [26].

In conclusion, we propose that the use of a combination of WNT inhibitor (KY02111) and BMP stimulator (BMP12) along with the optimal incubation time can enhance tenogenic differentiation more than BMP12 alone. Further study is required to improve the differentiation protocol, including

the determination of the optimal concentrations of KY02111 and BMP12, investigation of the effect of diverse cell lines, and comparison of KY02111 with other molecules.

Acknowledgments

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