Induction of chondrogenic morphogenesis in tissue culture using different combinations of transforming growth factor-β superfamily proteins *in vitro*

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To my family

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Zusammenfassung

HINTERGRUND: Die Regeneration von Knorpel war seit jeher eine Herausforderung für das Tissue Engineering. Ständig erneuerte Erkenntnisse über die Rolle der Transformation der Proteinfamilie des Wachstumsfaktors Beta (TGF-β), die für verschiedene grundlegende biologische Prozesse bei der Gesundheit und Regeneration des Knorpels von entscheidender Bedeutung ist, haben neue Perspektiven für die Behandlung knorpelbedingter Erkrankungen eröffnet. In dieser Studie sollte untersucht werden, wie sich drei verschiedene Wachstumsfaktoren aus der TGF-β Supergenfamilie spezifisch auf das knochenmorphogenetische Protein 2 (BMP-2) auswirken. TGF-β_3; osteogenes Protein 1 (OP-1), allein, jedoch insbesondere in verschiedenen Kombinationen, einschließlich unterschiedlicher Anwendungsdauer, sollte die Induktion der Chondrogenese im Muskelgewebe von Ratten bewirken.

METHODEN: Es wurde Bauchmuskelgewebe von Ratten verwendet. Um zu überwachen, wie sich die Anwesenheit von Morphogen auf die Chondrogenese auswirkt, wurden in der „Entzugsstudie“ zwei Stimulationsarten untersucht. Hierbei handelte es sich um eine kontinuierliche Applikation relevanter Morphogene und ihrer Kombinationen über die gesamte Dauer der In-vitro-Kultur oder eine einmalige Applikation nur für 48 h. Die Nachweise wurden am Tag 7, 14 und 30 mittels Immunhistochemie (IHC), histologischer Färbung (Alcianblau-Färbung) und quantitativer Reverser Transkriptase-Polymerase-Kettenreaktion (qRT-PCR) durchgeführt. Aggrecan wurde als Zielantigen in der IHC behandelt. Die relativen

Genexpressionsniveaus wurden analysiert, um das Überleben des Modells und der Chondrogenese zu bestätigen, *einschließlich des vaskulären endothelialen Wachstumsfaktors A* (*VEGF-A*), *Kollagen Typ IV alpha 1* (*Col4α1*), *geschlechtsbestimmende Region Y (SRY) -box 9* (*SOX9*), *Aggrecan* (ACAN), *Kollagen Typ II alpha 1* (*Col2α1*), *Kollagen Typ X alpha 1* (*Col10α1*), *Kollagen Typ I alpha 1* (*Col1α1*) und *alkalische Phosphatase* (*ALP*).

ERGEBNISSE: Die Ergebnisse der qRT-PCR zeigten, dass die Hochregulierung der Genexpression für die kontinuierlichen Versuchsgruppen signifikant höher war als die der einzelnen 48h-Stimulationsgruppen. Die Gruppe mit BMP-2 allein zeigte am Tag 7 kontinuierlich die höchsten relativen Expressionsniveaus, ausgedrückt als chondrogen-verwandte Gene. Positive Reaktionen wurden bei der Alcianblau-Färbung und IHC mit semi-quantitativer histomorphometrischer Analyse beobachtet, die eine Korrelation zu der der Genexpressionsmuster zeigten.

SCHLUSSFOLGERUNGEN: In dieser chondrogenen Induktionsstudie erwies sich Muskelgewebe als brauchbares Modell. Die Anwendung von Mitgliedern der TGF-β Supergenfamilie allein oder in Kombinationen induzierte die Chondrogenese in diesem Gewebemodell, wobei die Ergebnisse darauf schließen lassen, dass die Hyalinknorpel-Chondrogenese auf der Grundlage der Col2α1 Expressionsmuster entwickelt wurde. Obwohl in diesem Projekt versucht wurde, mithilfe der Rückzugsstudie ein wirtschaftlicheres Induktionsschema zu erhalten, wurde gezeigt, dass die einmalige

Stimulation mittels eines Wachstumsfaktors nicht ausreicht, um die entsprechende Reaktion hervorzurufen. Dies deutet stark darauf hin, dass eine kontinuierliche Stimulation erforderlich ist. Die Ergebnisse in dieser Hinsicht müssen jedoch mit Vorsicht interpretiert werden, da klar ist, dass ein einzelnes Morphogen eine begrenzte räumliche und zeitliche Wirkung hat, wenn das Vorhandensein des entsprechenden komplementären löslichen Signals (der entsprechenden komplementären löslichen Signale) zum richtigen Zeitpunkt vorliegen muss zur Gewährleistung einer ordnungsgemäßen und dauerhaften biologischen Reaktion bestimmter Pfade im Laufe der Zeit. Dies wurde durch BMP-2 veranschaulicht, dass allein die Chondrogenese initiieren konnte, jedoch bei Zugabe in Kombination mit TGF-β_3 und / oder OP-1 inhibiert wurde. Während BMP-2 anfänglich die Chondrogenese stimulierte, konnte es die relevante Reaktion im mittleren und späten Stadium der chondrogenen Induktion nicht aufrechterhalten, wobei TGF-β_3 und OP-1 zur Aufrechterhaltung der Knorpel-Tissue-Engineering-Reaktion erforderlich waren. Obwohl es noch Einschränkungen gibt, bieten die Experimente eine entscheidende Erkenntnis für das Tissue Engineering der TGF-β Supergenfamilie und liefern neue Erkenntnisse und Strategien für die Herstellung von Hyalinknorpel für zukünftige klinische Anwendungen.

Summary

BACKGROUND: The regeneration of cartilage has always been a challenge for tissue engineering. Constantly renewed insights into the role of transforming growth factor-beta (TGF-β) supergene family of proteins, which are vital in several fundamental biological processes in cartilage health and regeneration, has opened up new prospects for the treatment of cartilage-related diseases. In this study, the aim was to investigate what the effect of three different growth factors from the TGF-β supergene family specifically [bone morphogenetic protein 2 (BMP-2); TGF-β_3; osteogenic protein 1 (OP-1)], alone but especially in varying combinations including application durations, would have on the induction of chondrogenesis in muscle tissue of rats.

METHODS: Abdominal muscle tissue from rats was utilized. To monitor what the effect of morphogen presence would have on chondrogenesis, the "withdrawal study", assessed two modes of stimulation. These were a continuous application of relevant morphogens and their combinations for the entire duration of the *in vitro* culture or a single application only for 48h. The detections were performed on day 7, 14 and 30 using immunohistochemistry (IHC), histological staining (alcian blue staining) and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Aggrecan was treated as the target antigen in the IHC. The relative gene expression levels were analyzed to confirm the survival of the model and the chondrogenesis, including *vascular endothelial growth factor A* (*VEGF-A*), *collagen type IV alpha 1* (*Col4α1*), *sex-determining region Y* (*SRY*)*-box 9* (*SOX9*), *aggrecan* (*ACAN*), *collagen type II*

alpha 1 (*Col2α1*), *collagen type X alpha 1* (*Col10α1*), *collagen type I alpha 1* (*Col1α1*) and *alkaline phosphatase* (*ALP*).

RESULTS: The results of the qRT-PCR showed that the up-regulation in gene expression for the continuous experimental groups was more significant than that of the single 48h stimulation groups. The group with BMP-2 alone continuously presented the highest relative expression levels on day 7, in terms of the chondrogenic-related genes. Positive reactions were observed in the alcian blue staining and IHC with semi-quantitative histomorphometrical analysis showing a correlation to that of the gene expression patterns.

CONCLUSIONS: Muscle tissue was proven to be a viable model in this chondrogenic induction study. The application of members of the TGF-β supergene family, alone or in combinations, induced chondrogenesis in this tissue model, with results suggesting that hyaline cartilage chondrogenesis was being developed based on the *Col2α1* expression patterns. Although it was attempted to get a more economic-efficiency induction scheme using the withdraw-study in this project, it was shown that single stimulation of a growth factor was insufficient to evoke the relevant response, strongly suggesting that a continuous stimulation is necessary. However, the results in this regard have to be interpreted with care as it is clear that a single morphogen has a limited spatial and temporal effect where the presence of the appropriate corresponding complementary soluble signal(s) needs to be present at the correct time to ensure a

proper and sustained biological reaction of specific pathways with time. This was exemplified by BMP-2 that on its own was able to initiate chondrogenesis, yet when added in combination with TGF-β_3 and/or OP-1 was inhibited. However, while the BMP-2 initially stimulated chondrogenesis, it could not maintain the relevant reaction in the middle and late stages of chondrogenic induction, where TGF-β_3 and OP-1 were necessary to maintain the cartilage tissue engineering reaction. Although limitations still exist, the experiments provide a crucial realization in the TGF-β supergene family tissue engineering prospect and deliver novel awareness and strategies in producing engineered hyaline cartilage for future clinical applications.

1. Introduction

Regeneration of cartilage remains a challenge for tissue engineering. This introduction summarizes and reviews the current status of cartilage regeneration research in the light of relevant literature. At the same time, the role of the transforming growth factor-beta (TGF-β) supergene family of proteins in inducing chondrogenic morphogenesis are briefly described to illustrate the significance of this study.

1.1 Cartilage and cartilage injure

The skeletal system contains various types of tissue: bone, cartilage, muscle and fat, which are all derived from common mesenchymal progenitors[1]. Chondrogenesis is the initial process of skeletal development in embryogenesis of most long bones, which in which the formation of a cartilage anlage is first laid down that is eventually transformed into bone via the process of endochondral ossification. During this phase, mesenchymal cells are recruited and differentiate into chondroblasts; subsequently, the extracellular matrix (ECM) is formed by molecules, such as aggrecan and collagen type II, secreted by mature chondrocytes[2,3]. Hence, cartilage can be divided into three types according to its structure and function formed by different components. Hyaline cartilage is the predominant type in humans which forms all articular surfaces, with a specialized type of hyaline cartilage existing in the epiphyseal plate[4,5]. Fibrocartilage is a transitional type between connective tissue and cartilage, which is often distributed in the connection between intervertebral disc, glenoid, pubic symphysis and the attachment of the tendon, capsule as well as the ligament on articular cartilage[6,7]. The

last one is the elastic cartilage, which is present in the auricle and epiglottis[8,9]. For load-bearing tissue, the relations between structure and function should be comprehended. Besides the extracellular water (66-78%), proteoglycans, collagen and additional specialized proteins, which constitute the cartilage matrix predominantly[10], collagen type I and elastin are the unique components for fibrocartilage and elastic cartilage, respectively[11]. Different components make different types of cartilage play different roles. The primary mechanical function of fibrocartilage and elastic cartilage is tension, but for hyaline cartilage is compression.

A review of 31,516 knee arthroscopies demonstrated that 63% of patients had chondral injury[12]. The pathological changes caused by mechanical injury or degenerative pathologies often lead to cartilage dysfunction, further resulting in joint effusion, pain and degenerative arthritis[13,14]. Because cartilage lacks nerves as wells as blood vessels and receives its nutrition solely by diffusion[15-17], once it is damaged, its self-repair ability is negligible[18-23]. Take the mechanical injuries of articular cartilage as an instance; nearly all damages resulting from forces applied to the cartilage and differ in the extent and type of tissue damage, leading to various injuries[24-26]. According to the extent of tissue damage, it can be divided into three types[27,28]: 1) cartilage injury without tissue disruption; 2) chondral fissures, flaps or fractures; and 3) osteochondral fractures. Each type stimulates a different response and raises a different problem for repair. For example, due to the lack of vessels, cartilage damage alone does not cause inflammation; however, when the disruptions extend through cartilage into subchondral bone[29-31], the

inflammatory reactions and the initiation of fracture repair can be triggered because of the damage to bone vessels[32,33]. Cartilage injury is a frequent occurrence and may have significant consequences. However, compared with other musculoskeletal injuries, our understanding of cartilage injury is still limited.

1.2 Cartilage repair and regeneration

Articular cartilage does not heal itself or only partially under certain biological conditions[18-23]. Numerous clinical and biological attempts have been made to induce a significant healing response within mature articular cartilage to reconstruct and repair tissue structurally including functionally[34,35]. Although conservative treatment is of great significance for cartilage injury, surgical strategies are also developing and innovating. Surgical interventions can be classified into two categories based on the involving of active biologics or not[36]. The commonly used management without active biologics participation included: lavage and arthroscopy[37], debridement[38], abrasion chondroplasty[39], shaving[40,41], laser abrasion/laser chondroplasty[42], microfracture technique[43-45], pride drilling[46] and spongialization[47]. However, the efficacy of these clinical methods was controversial. For example, some studies showed that therapeutic effects of lavage could persist for a year or more[48], but there were also views that patients undergoing this therapy did not obtain substantial relief from clinical symptoms, such as pain[49].

Current cartilage restorative techniques with active biologics mainly include fresh osteochondral allografts[50], cultured chondrocyte implantation[51] and osteochondral autografts [52]. Gross et al.[53,54] first attempted to apply fresh osteochondral allografts to offset the segmental loss of bone and cartilage in the treatment of osteoarthritis in the knee, with promising results. After a 40-month follow-up of 67 patients, Davidson et al.[55] found that both the International Knee Documentation Committee and SF-36 scores were significantly improved compared with preoperative values and nearly normal International Cartilage Repair Society scores were obtained. A circular socket was created first in osteochondral autograft and then harvested using a circular tube osteotome which resulted in the limitation of this technique that was the amount of available donor material and harvest-related morbidity[56-60]. Previous cell transplantation and current matrix-assisted scaffold techniques provided cell-based options for repairing cartilage defects[61-63]. However, this two-stage cartilage restoration technique remains an issue that creates substantial cost and inconvenience in the clinic, especially the intervening period of cell culture[64,65]. In addition, chondrocytes tend to dedifferentiate toward a fibroblastic phenotype when cultured *in vitro*, which was presented by Eric et al [66]. In their cell culture study, the average *collagen type II alpha 1* (*Col2α1*) / *collagen type I alpha 1* (*Col1α1*) ratio decreased four orders of magnitude ($p < 0.0001$) over only two passages, indicating a rapid change in phenotype from chondrocytic to fibroblastic. Although characterized chondrocyte implantation has been developed, the clinical benefits still require verification[67,68].

In the past two decades, tissue engineering has attempted to use living and functional structures to heal damaged or defective structures[69]. Articular cartilage was the most promising first-generation product because of its homogeneous structure, fewer cell types and nearly two-dimensional characteristics[19]. However, the regeneration of

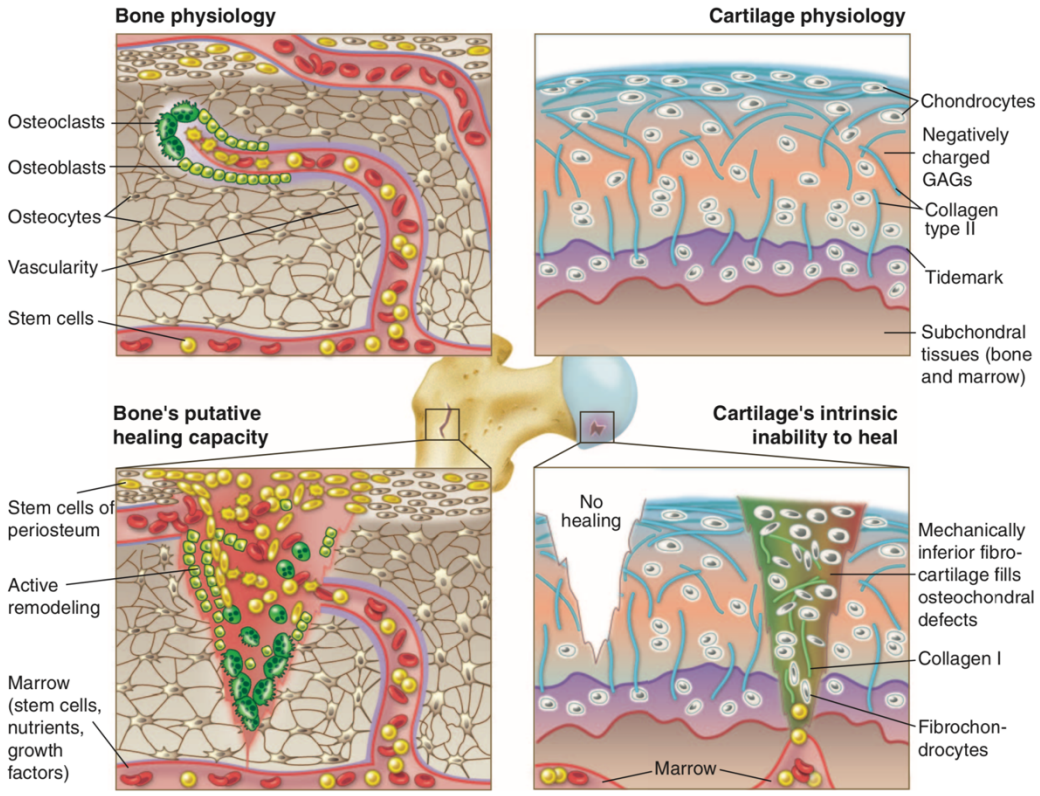

Figure 1. The physiology and putative healing capacity of bone and cartilage. Differences in the physiological environment, metabolic rate, and cell composition of bone and cartilage have significant effects on the self-repairing ability and tissue engineering potential. Cartilage's hypocellularity and lack of nutrient supply preclude healing, while bone integrates rapidly, even with metal. Thus, in contrast to bone's healing ability, cartilage requires more robust exogenous interventions to achieve satisfactory regeneration. (Taken from Huey et al 2012[19])

cartilage, the tissue type of which is simple, was not as successful as predicted and bone was likely to be earlier regenerated by tissue engineering despite its more complex composition due to the characteristics of cell activity and rich blood vessels.[70-72]. Only a few and low metabolic activity cells exist in the cartilage, which limits the production of engineered cartilage for clinical use[10,73] (**Figure 1, taken from Huey et al. 2012**[19]).

Stem cells or terminally differentiated cells which are suitable for cartilage regeneration, share the ultimate goal of producing tissue-specific ECM, followed by the consideration of the readily available and easily inducible properties[74]. Mesenchymal stem cells (MSCs) have multiple differentiation potential, including chondrocyte, fibro-chondrocyte and hypertrophic chondrocyte, which is an ideal resource for cartilage regeneration[75]. However, studies indicated that although cell transplantation has shown short-term clinical outcomes, such as the enhancement of clinical knee-function scores, after two years of follow-up, the results had become worse. The lack of functional mechanical properties failed the repair strategy[76,77]. In addition, the use of scaffolds in cartilage regeneration is controversial. *In vitro*, studies on cartilage regeneration focus more on the design of scaffolds aim to better organization and differentiation[78,79]. However, the following challenges, such as the biodegradation rate of scaffolds, by-products and harsh chemicals involved with scaffold fabrication, encouraged researchers to promote the development of scaffold-free technology[80]. Although currently, the cartilage regeneration continues to be elusive, the implementation of new approaches has provided the possibility for the development of alternate cartilage regeneration technologies, such as the use of various growth factors [81,82].

1.3 TGF-β supergene/protein family

The TGF-β supergene family of proteins is vital for several fundamental biological processes, including embryonic development and organ morphogenesis[83,84]. The

renewed constantly insights into the role of the TGF-β superfamily in cartilage health and regeneration opens up new prospects for the treatment of cartilage-related diseases.

1.3.1 Overview of TGF-β supergene/protein family

The name, TGF-β superfamily, was taken from the first isolated member (TGF-β_1)[85]. However, the name is misleading because TGF-β_1 was proved to inhibit the proliferation of many cell lines and the original "transformation" function may be related to matrix production and synergistic effects with other growth factors[86,87]. The members of TGF-β superfamily increased from 17 in 1990 to at least 30 nowadays. Based on the sequence similarity and function, they can be divided into two subfamilies, which are TGF-β and bone morphogenetic protein (BMP) subfamily[88,89]. The TGF-β subfamily consists of the three TGF-β isoforms, Activin (A and B), Nodals, myostatin and Mullerian inhibiting substance, wherein the BMP subfamily BMPs 2, 4-10 and the growth and differentiation factors (GDFs) are included.

The cascade reactions in TGF-β signaling are evoked by the binding and assembling between ligands and the receptor complex on the cell membrane, including serine/threonine kinase types I and II receptors[89,90] and the activated type II receptors phosphorylate type I receptors[91]. Type I receptors are also known as activin receptor-like kinases (ALK) and seven types have been found so far[92]. ALK 1, 2 and 3 are bound with BMPs, while ALK 4, 5 and 7 are bound with TGF-βs[93]. The phosphorylation not only activates receptors but also provides binding sites for downstream signaling

mediators, the receptor-regulated SMAD proteins (R-SMADs)[94]. The traditional view of TGF-β superfamily signaling for BMPs is through R-SMADs 1, 5 and 8, while the members of the TGF-β subfamily transduce signals through R-SMADs 2 and 3[93]. Upon receptor-mediated phosphorylation, the specific R-SMADs then form heteromeric complexes with SMAD 4, a common intracellular mediator shared by the entire TGF-β superfamily[95]. The complexes are translocated into the nucleus, induce the transcriptional responses combining with other transcription factors[91], which is thought to be the canonical signaling pathway. The SMADs 6 and 7 (I-SMADs) act as inhibitors in the BMPs and TGF-βs signaling cascade[91]. In addition, BMPs/TGF-βs signals can also be transmitted through non-canonical, SMAD-independent avenues, the mammalian target of rapamycin (mTOR) pathways and etc[96,97].

1.3.2 TGF-βs/BMPs signaling in cartilage development and maintenance

TGF-βs play a vital role during the entire differentiation progress, including the regulation of condensation, proliferation, terminal differentiation and maintenance[92,98,99]. A large amount of *in vitro* and *in vivo* evidence indicated that TGF-β signaling promotes mesenchymal condensation, joint formation and regulate the physiology of postnatal/articular cartilage and growth plate[100,101]. Shintani et al.[102] proved the TGF-β_1 inhibit hypertrophic differentiation in bovine synovial explants and other studies also demonstrated that the TGF-β_3 arrested the terminal differentiation in MSCs chondrogenesis[103,104]. These data indicated that TGF-βs initiated the chondrocyte differentiation but represses the terminal hypertrophy. In terms of the growth plate,

SMAD 2 and 3, which are mediated by TGF-βs, play distinct roles throughout the entire period. SMAD 2 is expressed at a relatively higher level in proliferative and pre-hypertrophic chondrocytes, whereas SMAD 3 is predominant in pre-hypertrophic and hypertrophic chondrocytes[105]. Depletion of SMAD 3 in chondrocytes resulted in the progressive articular cartilage degeneration, which confirmed the significance of SMAD 3 in cartilage maintenance[105]. However, the function of SMAD 2 in cartilage *in vivo* is still unclear. On the one hand, SMAD 3 are directly bound to DNA for transcriptional regulation, whereas SMAD 2 first require to interact with SMAD 3 or other transcription factors[106]. SMAD 2 may partially compensate for SMAD 3 in preventing chondrocyte terminal differentiate[107].

BMPs are active substances derived from bone, which are identified by inducing ectopic bone and cartilage formation *in vivo*[108], while the mechanism has been the subject of intense research for the last three decades. Derynck et al.[109] elucidated the framework of BMP signal transduction pathway, enabling researchers to enter the molecular era of BMPs-induced cartilage morphogenesis. Other studies have shown that BMPs directly regulate chondrocyte-specific genes and transcription factors, allowing BMPs to establish connections with chondrogenesis[110,111]. Additionally, the proliferation in the growth plate raised by BMPs was first described by Brunet et al.[112] and the intersection with other pathways was also observed. Among these BMPs, BMP-2 is the first molecule to be clearly described to induce cartilage and bone formation and accumulating evidence suggests that the effect raised by BMPs is the result of a

combination of a set of BMP-2[108]. BMP-7, marketed as osteogenic protein 1 (OP-1), was the first growth factor commercialized for the treatment of osteoarthritis, although the effort was halted and the ability of OP-1 to repair cartilage is reflected in the synthesis of proteoglycans, collagen and hyaluronic acid[113].

1.4 Muscle tissue *in vitro* model

Stem cells are most widely used for tissue regeneration, especially for cartilage formation, as they can differentiate into different types of mature cells under appropriate conditions[114,115]. The drawbacks of the autologous stem cells transplantation in the clinic have been reviewed above, including the time-consuming and the expensive procedure which may not lead to a successful regeneration[116,117]. As such, alternative sources and strategies are needed.

The cartilage, bone and muscle are intimately connected tissue and contribute to the coordinated interplay in their development, function and aging[93,118,119]. Hence, the use of tissue instead of isolated cells provides a new option to develop more appropriate clinical applications. Skeletal muscle is highly adaptable, which is critical in determining the overall health, mobility and athletic performance of an individual. The musculoskeletal system can alter fiber size, functional capacity and metabolism in response to exercise, injury, illness, including other physiological stimuli[119,120]. Many factors make muscle tissue a candidate that can serve as a chondrogenic regeneration

implant. The muscle tissue model provides a familiar internal milieu and structural scaffold for cell survival and in the sense of a clinical application can also lower the donor site morbidity[121]. The muscle tissue graft contains a variety of cells with mesenchymal multipotencies, such as satellite cells, muscle-derived MSCs and even myoblasts [122-124]. In terms of osteogenic regeneration, several studies demonstrated that muscle-derived stem cells could differentiate into the osteogenic lineage *in vitro* and *in vivo* and improve bone healing[125,126]. Betz et al. attempted to use muscle tissue fragments to achieve regeneration in the osteochondral defect[127-130]. When compared with autologous bone transplantation, gene modification of tissue surface cells caused by direct contact with gene vectors and subsequent implantation resulted in similar biomechanical stability and new bone volume[127-130]. Li et al.[131] identified and characterized the chondrogenic progenitor cells in the fascia of postnatal skeletal muscle by analyzing the surface markers, proliferation rate and chondrogenic ability. Moreover, the application of muscles with fascia in the clinic can make the graft have a better fixation. All of these conditions are relevant arguments such that more effort has to be invested in determining the benefits in muscle culture combined with the induction of chondrogenesis.

1.5 Experiment design, aim and hypotheses

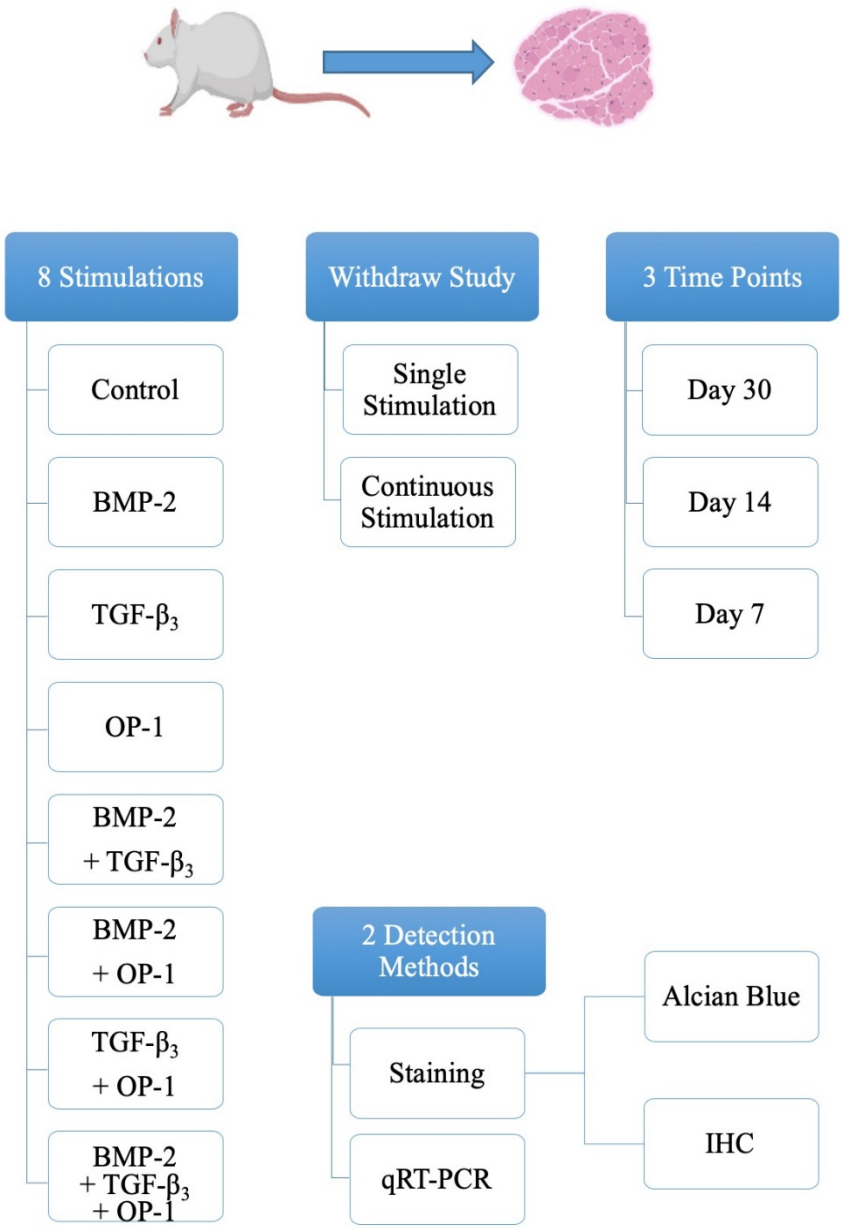

Figure 2. The outline of *in vitro* chondrogenesis induction using muscle tissue. Three different growth factors from the TGF-β superfamily were used, alone or in combinations to induce chondrogenesis in rat abdominal muscle tissue. Concerning the withdrawal study, two modes of stimulation were applied, which were a continuous application or a single application for an initial 48h. The detections were performed on day 7, 14, and 30, including IHC, histological staining, and qRT-PCR. TGF-β_3: transforming growth factor-beta 3; BMP-2: bone morphogenetic protein 2; OP-1: osteogenic protein 1. IHC: immunohistochemistry; qRT-PCR: quantitative reverse-transcriptase real time polymerase chain reaction.

1.5.1 Experiment design

This study attempted to detect the chondrogenic induction capacity of three different growth factors from TGF-β superfamily (TGF-β_3, BMP-2 and OP-1), alone or in different combinations. The experimental substances were fresh rat abdominal muscle tissue. To monitor the effect of morphogen presence would have on chondrogenesis, the withdrawal study, assessed two modes of stimulation. These were a continuous application of relevant morphogens and their combinations for the entire duration of the in vitro culture or a single application only for 48h. Results were assessed using immunohistochemistry (IHC), histological staining and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Aggrecan was treated as the target antigen in the IHC, as it is a key molecule in chondrogenesis. Alcian blue staining was also performed to indicate the presence of proteoglycans. The relative gene expression levels were analyzed to assess survival, including *vascular endothelial growth factor A* (*VEGF-A*) and *collagen type IV alpha 1* (*Col4α1*) and chondrogenesis, *including sex-determining region Y (SRY)-box 9* (*SOX9*), *aggrecan* (*ACAN*) and *Col2α1*. In addition, the relative expressions of *collagen type X alpha 1* (*Col10α1*), *Col1α1* and *alkaline phosphatase* (*ALP*) were also detected to determine what type of cartilage matrix was being developed (**Figure 2**).

1.5.2 Aims and Objectives of the study

The central aim of the study was to investigate how three specific morphogens (TGF-β_3, BMP-2 and OP-1), known to be involved in chondrogenesis, would have on

inducing this process in muscle tissue when these where either applied alone or in different combinations with each other, i.e. BMP-2 + TGF-β_3, BMP-2 + OP-1, TGF-β_3 + OP-1 and BMP-2 + OP-1 + TGF-β_3. Additionally, a subsequent aim was to determine how long morphogens would need to be active within the culture to maintain a chondrogenic response. To this end, the growth factors were applied for either 48h or the entire duration of the culture period. Finally, since there were limited studies using muscle tissue as the experimental model, the last aim was to validate the survival of muscle model *in vitro* after growth factor application and if this tissue type could be used to be transformed into cartilage material.

Objectives:

1. Chondrogenesis induction by the different morphogens alone or their combinations, as well as their application duration, were monitored using IHC using aggrecan in conjunction proteoglycan production (alcian blue staining) to determine what the type of tissue morphogenesis was taking place.
2. Gene expression patterns were assessed using qRT-PCR to validate that muscle tissue was thriving (*VEGF-A*). Also, the effect of the various morphogens and especially their combinations were evaluated to see what the modulatory or synergistic aspect was.

1.5.3 The hypotheses of this study

The hypothesis was that a single dose stimulation for 48h would be sufficient to induce chondrogenic morphogenesis but cannot maintain it. Through a continuous application of the selected morphogens, the chondrogenic response can be maintained. Another hypothesis was that the more growth factor involved, the more stable chondrogenesis would be triggered. Additionally, muscle models can survive *in vitro* and are a suitable tissue type for inducing hyaline chondrogenesis.

2. Methods

2.1 Acquisition of sample

Four F-344 adult rats (Charles River Sulzbach, Germany) were killed by an overdose of isoflurane (Abbot, Chicago, USA). Tissue harvest procedures were conducted according to the rules and regulations of the Animal Protection Laboratory Animal Regulations (2013), European Directive 2010/63/EU and approved by the Animal ethics research committee (AESC) of the Ludwig Maximillian's University of Munich (LMU), Bavaria, Germany Tierschutzgesetz §1/§4/§17 (https://www.gesetze-im-internet.de/tierschg/TierSchG.pdf) with respect to animal usage for pure tissue or organ harvest only. Under sterile conditions, fresh abdominal muscle tissue slab was excised and washed twice in Dulbecco phosphate-buffered saline (PBS; Biochrom GmbH, Berlin, Germany). The tissue slab was then immersed in the Alpha medium (Biochrom GmbH) supplement with 2% penicillin and streptomycin (P/S, Biochrom GmbH) for 30 minutes, rinsed briefly again in PBS after which it was placed finally in culture medium (Alpha medium, 1% P/S and 0.02 mM/mL L-glutamine, Biochrom GmbH). Then using a 4mm biopsy punch (PFM medical, Cologne, Germany), 4mm in diameter muscle pieces were harvested from the rectus abdomins muscle slab. In a total of 576 biopsies were harvested and then placed in 96-well Nunc culture plates (Thermo Fischer Scientific, Denmark) with normal culture medium for 48h to allow for tissue recovery.

2.2 Tissue culture

After the 48h recovery period, the medium was replaced by relevant differentiation medium, which was taken as day 0 of the culturing process. The differentiate medium was the culture medium supplemented with three different growth factors alone or in varying combinations: 50 ng/mL Recombinant Rat Bone morphogenetic protein 2 (BMP-2, CUSABIO, USA), 50 ng/mL Recombinant Transforming Growth Factor Beta 3 (TGF-β_3, Cloud-Clone Corp., USA); 50 ng/mL Recombinant Bone Morphogenetic Protein 7 (BMP-7 or OP-1, Cloud-Clone Corp., USA); 50 ng/mL BMP-2 + 50 ng/mL TGF-β_3; 50 ng/mL BMP-2 + 50 ng/mL OP-1; 50 ng/mL TGF-β_3 + 50 ng/mL OP-1; 50 ng/mL BMP-2 + 50 ng/mL TGF-β_3 + 50 ng/mL OP-1. All the differentiation groups were compared to control group cultured in culture medium without any growth factors. In terms of the withdrawal study, the medium in half experimental groups was replaced with the normal culture medium after 48h of their first stimulation of growth factors. The medium was changed every two days as required. All samples were cultured at 37℃ in a 5% CO_2 humidified incubator and harvested on day 7, 14 and 30. Half of the samples were harvested and then frozen in the liquid nitrogen (N_{liq}) immediately. They were kept in the -80℃ until used for qRT-PCR. Half of the samples were fixed in 30% formalin (Microcos GmbH, Germany) for 24h, dehydrated in a Gewebeprozessor Entwässerungsautomat STP 120 unit (Thomas-medical e.U., Maishofen, Austria) and then processed for paraffin wax embedding.

2.3 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

In order to obtain more accurate relative gene expression results, pre-experiments and corresponding optimization for each step were conducted, including the determination of reference genes, reaction volume as well as ratio, gradient tests of optimal temperature and substrate concentration. The qBase+ software (https://www.qbaseplus.com/) was applied for the normalization. The final results were presented in the form of calibrated normalized relative quantities (CNRQ) values and analyzed statistically.

2.3.1 Primer design

PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) was used to find information about genes of interest. Search parameters were set in the "Nucleotide" database accordingly to gene name with the restriction conditions of species, molecule and organisms limited to "Animal", "mRNA" and "*Rattus norvegicus* [rodents]", respectively. Then the coding sequences (CDS) were saved through FASTA interface (FASTA was a suite of programs for searching nucleotide or protein databases with a query sequence), so sequence coding for amino acids in the protein of genes of interest was obtained.

Primers were designed and evaluated utilizing IDT website (https://eu.idtdna.com/site). CDS were assessed for viable primers using PrimerQuest Tool. After entering the sequence name, "CUSTOM DESIGH PARAMETERS" was selected and the following

settings were applied:

1) The result to return was 50.

2) The Primer Melting Temperature (T_m) containing the minimum, optimum and maximum melting temperatures (Celsius) for a primer oligo were set as 59, 60 and 61.

3) The primer GC% containing minimum, optimum and maximum percentage of Gs and Cs in any primer generated by PrimerQuest were set as 47, 50 and 53.

4) The primer sizes containing the minimum, optimum and maximum size of the desired primer(s) were set as 16, 18 and 20.

5) The amplicon sizes containing the minimum, optimum and maximum size for the desired amplicon were set as 150, 160 and 210.

Generated possible primers were subsequently screened using the OligoAnalyzer Tool to select the best primers that would deliver specific amplification of the desired gene(s) region without generating artifact amplifications. The following Gibbs standard free-energy change (ΔG) scores were looked for in generated primers: a hairpin structure $\Delta G > -2$ and a self-dimer structure $\Delta G > -5$, with the pairs of both dimers < 3. Also, hetero-dimer non-specific amplifications between forward and reverse primers for a gene required limited to below 3 with $\Delta G > -6$.

Primers were scaled from best to worst accordingly. After a temperature gradient optimization step using the best possible primer pair, providing information about the optimum amplification temperatures to use during the qRT-PCR, the relevant amplified PCR products were purified with the Mini Elute PCR Purification Kit (Qiagen, Hilden,

Germany) and primer specificity confirmed by Sanger sequencing (GATC Biotech, Cologne, Germany) in conjunction with nucleotide mega-blast analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch)[132,133].

2.3.2 The determination of the optimal reference genes by GeNorm

To optimize the normalization, geNorm (http://medgen.ugent.be/wjvdesomp/genorm/) was used to find the most stable reference genes and the number required to generate accurate gene expression data[134]. The primers of eight candidate genes were designed according to the Primer design method (**Section 2.3.1**) *TATA-binding protein* (*TBP*), *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *RNA polymerase II subunit e* (*POLR2e*), *ribosomal protein lateral stalk subunit P0* (*RPLP0*), *succinate dehydrogenase complex flavoprotein sub-unit A* (*SDHA*), *ribosomal protein L13α* (*RPL13α*), *RNA-28S ribosomal 4* (*RNA28S4*) and *actin beta* (*ACTB*) (**Table 1**) were tested. After all the samples were harvested, the relative gene expressions of eight candidate genes in all samples were obtained by qRT-PCR (mean of duplicate detections) in the form of the cycle threshold Value (Ct; more correctly called cycle quantification, Cq). The delta-Cq value was calculated based on the original Cq value of each gene according to the formula:

$$\Delta \mathrm{Cq} = 2^{(\mathrm{Cq_{min}} - \mathrm{Cq_{sample}})}$$

After the expression data matrix was loaded into GeNorm, two charts were generated. The first graph presented the genes, ranked, according to increasing expression stability, with the most stable genes beginning from the right of the chart. The second chart

indicated the results of the pairwise variation (V) between two sequential normalization factors containing an increasing number of genes revealing the optimal quantity of the reference gene. The lowest V value was set as the cut-off value below which the

Table 1. Gene primers for *Rattus norvegicus* with accession number and sequence

	Gene	Accession Number	5' – 3' sequence	3'- 5' sequence
Reference genes	*TBP*	BC081939.1	TAACCCAGAAAGTCGAAGAC	CCGTAAGGCATCATTGGA
	GAPDH	BC083511.1	CATGGGTGTGAACCATGA	TGTCATGGATGACCTTGG
	POLR2e	BC158787.1	GACCATCAAGGTGTACTGC	CAGCTCCTGCTGTAGAAAC
	RPLP0	BC001834.2	CAACCCAGCTCTGGAGA	CAGCTGGCACCTTATTGG
	SDHA	NM_130428.1	GCGGTATGAGACCAGTTATT	CCTGGCAAGGTAAACCAG
	RPL13α	NM_173340.2	TTTCTCCGAAAGCGGATG	AGGGATCCCATCCAACA
	ACTB	NM_031144.3	AGCTATGAGCTGCCTGA	GGCAGTAATCTCCTTCTGC
	RNA28S4	NR_145822.1	GCGGCCAAGCGTTCATA	CCTGTCTCACGACGGTCTAA
Genes of interest	*Col1α1*	NM_053304.1	GGTGACAGAGGCATAAAGG	AGACCGTTGAGTCCATCT
	Col2α1	NM_012929.1	ATCCAGGGCTCCAATGA	TCTTCTGGAGTGCGGAA
	Col4α1	NM_001135009.1	CTGGGAATCCCGGACTT	GGGATCTCCCTTCATTCCT
	Col10α1	XM_001053056.7	CCAGGTCTCAATGGTCCTA	ATTTCCTCACGGACCTGT
	ACAN	NM_022190.1	CAAGTGGAGCCGTGTTT	TTTAGGTCTTGGAAGCGAG
	ALP	NM_013059.2	CGACAGCAAGCCCAAG	AGACGCCCATACCATCT
	SOX9	NM_080403.1	CCAGAGAACGCACATCAAG	ATACTGATGTGGCTGGTGG
	VEGF-A	NM_001317043.1	CTACCAGCGCAGCTATTG	GATCCGCATGATCTGCATAG

TBP: *TATA-binding protein*, *GAPDH*: *Glyceraldehyde 3-phosphate dehydrogenase*, *POLR2e*: *RNA polymerase II subunit e*, *RPLP0*: *Ribosomal protein lateral stalk subunit P0*, *SDHA*: *Succinate dehydrogenase complex flavoprotein sub-unit A*, *ROL13α*: Ribosomal protein L13*α*, *ACTB*: *Actin beta*, *RNA28S4*: *RNA 28S ribosomal 4*, *Col1α1*: *Collagen Type I Alpha 1*, *Col2α1*: *Collagen Type II Alpha 1*, *Col4α1*: *Collagen Type IV Alpha 1*, *Col10α1*: *Collagen Type X Alpha 1*, *ACAN*: *Aggrecan*, *ALP*: *Alkaline phosphates*, *SOX9*: *SRY (Sex Determining Region Y)-Box 9*, *VEGF-A*: *Vascular endothelial growth factor A*.

participation of more reference genes was redundant.

2.3.3 qRT-PCR

The whole qRT-PCR process was compliant with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines[135]. The primers of eight genes of interest were designed according to the primer design method described in Section 2.3.1. Genes selected for the study were: *VEGF-A*, *Col4α1*, *SOX9*, *ACAN*, *Col2α1*, *Col10α1*, *Col1α1* and *ALP* (**Table 1**). Harvested specimens designated for gene expression analysis were taken from -80℃ and under sterile RNase-free conditions homogenized into a fine powder using N_{liq} in conjunction with mortar and pestle. The modified RNA was then extracted using the RNeasy® Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany), with the specific steps shown in **Figure 3**. The quality and concentration of RNA were detected using NanoDrop™ Lite (Thermo Scientific) by spectrophotometry. Total RNA was kept at -80 ℃ until used. RNA was reverse transcribed using the QuantiTect Reverse Transcription cDNA Synthesis Kit (Qiagen). cDNA was kept at -20 ℃ until used. qPCR was performed on a LightCycler® 96 Instrument (Roche, Basel, Switzerland) in duplicate using 2x FastStart Essential DNA Green Master (Roche). The process of thermocycling included a 2 minutes denaturation step at 94 °C, 40 cycles containing a denaturation, annealing and extension step set at 95 °C for 10s, 60 °C for 15s and 72 °C for 30s, respectively; and a final extension at 72 °C for 5 minutes. The total volume of each reaction was 10μL and the ratio of each reactant was determined by a pilot study, containing 5μL Green Master, 0.6μL forward

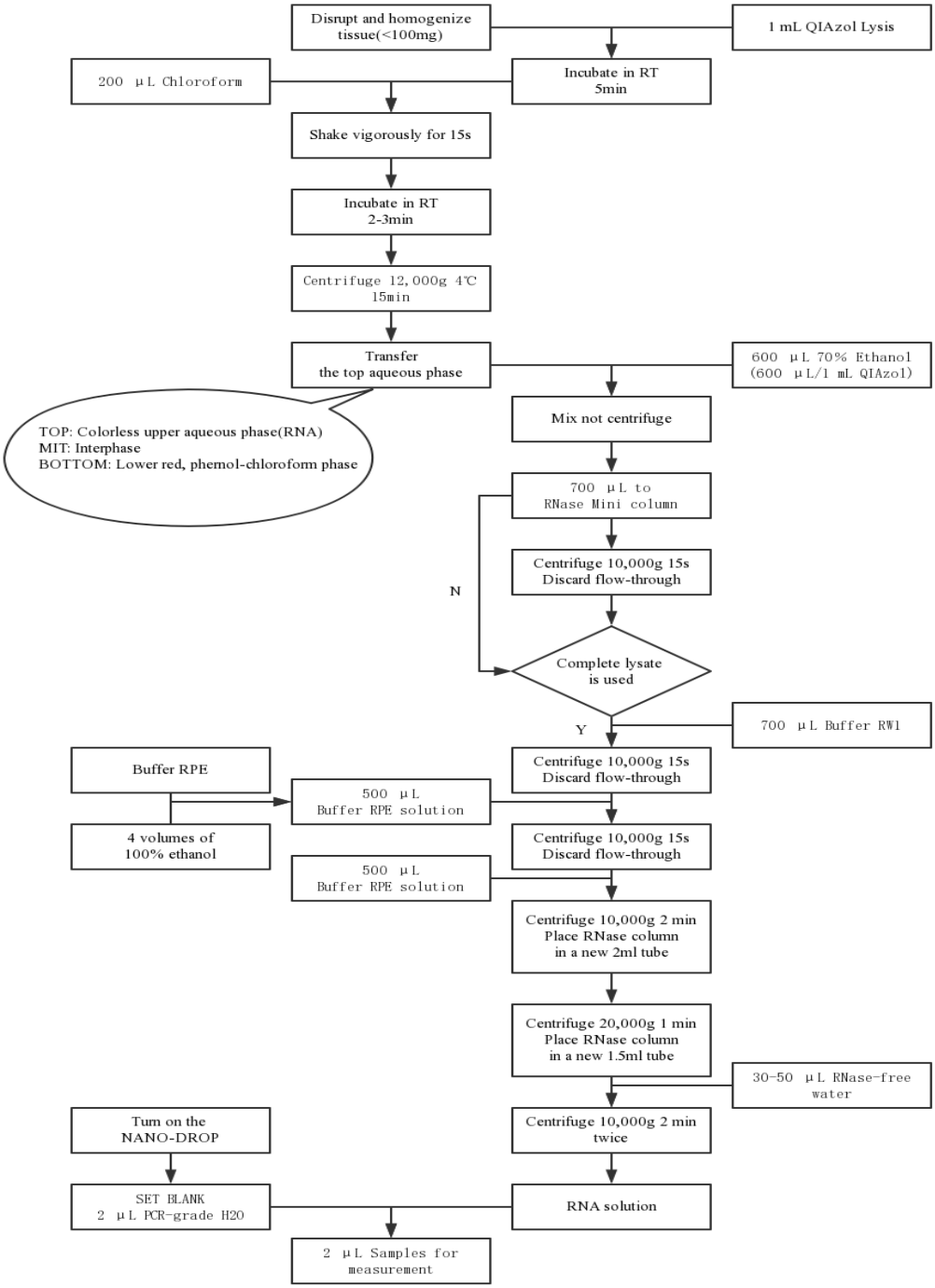

Figure 3. Flowchart of modified RNA extraction using a RNeasy® Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). QIAzol was used to lyse cells, and RNA integrity was maintained. Chloroform was added and centrifuged. The solution was divided into the aqueous phase, interphase, and phenol-chloroform phase. RNA exists in the aqueous sample layer. After washing with RW1 and RPE, DNA and protein in the sample were removed by precipitation.

primer (10µmol/L), 0.6µL reverse primer (10µmol/L), 2µL cDNA (5ng/µL) and 1.8µL

RNase-free water. The LightCycler® software (Roche) was used to generate Cq values of each gene in each sample.

To generate the CNRQ data, qBase+ was used utilized. Groups were normalized to the pre-determined reference genes, including fresh muscle tissue that reflects the base-line in CNRQ graphs.

2.4 Histologic analyses (Alcian blue)

The sample preparation process was described in **section 2.2** and 2µm-thick paraffin wax sections of specimens were mounted on Superfrost glass slides (Menzel, Braunschweig, Germany). Alcian blue staining was used to detect the presence of glycosaminoglycans in cartilages and other body structure; hence, the detection of mucopolysaccharides was accomplished by using the alcian blue- nuclear red stain kit. Briefly, sections were de-waxed prior to staining by first placing the section at 60 °C for 1h followed by immersion into xylene (SAV Liquid Production GmbH, Flintsbach am Inn, Germany), twice, for 10 and 5 minutes each. Re-hydration of the sections was achieved by decreasing ethanol concentrations (100%, 100%, 96%, 70% ethanol; each step 5 minutes; Apotheke Großhadern, Munich, Germany) into deionized (DI) water. After having been left in DI for 2 minutes sections were transferred to 3% acetic acid (Morphisto- Evolutionsforschung und Anwendung GmbH, Frankfurt am Main, Germany) for 3 minutes followed by Alcian Blue staining at pH 2.5 (Morphisto-Evolutionsforschung und Anwendung GmbH) for 30 minutes. Sections were then

transfer back to 3% acetic acid for 3 minutes, washed briefly for 5 min in DI water and subsequently counterstained in nuclear fast red solution (Morphisto-Evolutionsforschung und Anwendung GmbH) for 5 minutes. Sections were then briefly agitated in DI water for 1 minute and dehydrated via ascending concentrations of ethanol into xylene followed by cover-slip mounting using EUKITT resinous mounting medium (O. Kindler GmbH, Bobingen, Germany)[136].

Histological sections were analyzed and images were captured using a PreciPoint M8 microscope (PreciPoint, Freising, Germany) with integrated Viewpoint software (PreciPoint). Histomorphometric analysis was performed using the Image-Pro Plus software (version 6.0, Meyer Instruments, Inc.). The total area of the tissue section and the positive area (area stained blue) were measured and the ratios between them (positive area/ total area) were considered as the original semi-quantitative analysis data of each section [132,133].

2.5 Immunohistochemical analyses

The detection of aggrecan antigen, one of the most abundant macromolecules of articular cartilage, was accomplished through IHC. Washing buffer (WB) was prepared in advance, which was usually a mixture of 2.5L PBS and 2.5mL Tween (Merck Schuchardt OHG, Hochenbrunn, Germany). Prior to IHC, slides with sections were placed for 1h at 60 °C before being placed in xylene for two changes of 10 min each.

Specimens were then re-hydrated through ascending levels of (100%, 100%, 96% and 70% ethanol; 5min each step) into DI water. Sections were placed into 3% H_2O_2 for 5 min, washed by the WB and then placed in the DI water.

Rodent Decloaker (ZYTOMED SYSTEMS GmbH, Berlin, Germany) was diluted by DI water and concentrated at a ratio of 1:10 to make the antigen retrieval buffer. The appropriate buffer was added to the *etui* holding the sections and then put into a pressure cooker for antigen retrieval. After 15 minutes slides were left to cool down for about 30 minutes within a deactivated pressure cooker after which they were removed from the etui and placed at room temperature for another 15 minutes.

All incubations in the staining process were conducted in a humidified chamber to avoid drying of the sections. Dilutions of the primary antibody in antibody diluent (ZYTOMED SYSTEMS GmbH) were determined by testing a range, which was 1:150 for aggrecan (Biorbyt Ltd., Cambridge, United Kingdom). Slides were first for 2 minutes in WB. Two drops of Rodent block R (ZYTOMED SYSTEMS GmbH) were dropped onto the tissue sections and then washed for 2 minutes in WB twice. The primary antibody was then applied and left at room temperate for 1 hour at the room temperature. After primary antibody application the sections washed three times, 2 minutes each, WB after which three drops of Rabbit-on-Rodent HRP-Polymer (ZYTOMED SYSTEMS GmbH) were applied and incubated for 20 minutes after which the sections were washed in WB again 2 minutes each. Liquid Vina Green

chromogen and buffer (ZYTOMED SYSTEMS GmbH) were then applied to the slides (3 drops) and incubated for 10 minutes at room temperature. The slides were briefly rinsed with DI water.

Counterstaining was achieved by using haematoxylon for 30 seconds, after which slides were rinsed under running tap water for 30 minutes after which they were then dehydration through ascending grades of alcohol into xylene. Finally, sections were mounted using a cover-slip and EUKITT resinous mounting medium.

Images were captured and digitalized using a PreciPoint M8 research microscope and Viewpoint software. Semi-quantitative histomorphometrical analysis was performed using Image-Pro Plus software. The absorbance value of the incident light in the blank of the tissue piece was calibrated firstly and then the area of total tissue and the integrated optical density value (IOD) of the positive area were measured. Then the mean optical density value (MOD) of the positive area was calculated (MOD= IOD/ area), which quantify the intensity of immunostaining[137].

2.6 Statistical analysis

Statistical evaluations were performed on qRT-PCR and histomorphometric data of both histological and IHC material. GraphPad Prism (version 7.0, GraphPad Software, San Diego, USA) was utilized to generate statistical results. A one-way ANOVA with

Dunnet’s test was used to determine the statistical differences between different experimental and corresponding control groups. A one-way ANOVA with Tukey's multiple comparisons test was used to compare the mean of each group with the mean of every other group. A two-way ANOVA with multiple comparisons was mainly used to compare the difference between each experimental group with a single and continuous application at the same time point and the changes of each experimental group over time, simultaneously. Statistical significance was defined at $p<0.05$.

3. Results

3.1 Gene expression

Eight candidate reference genes were assessed in all samples using qRT-PCR where finally, *TBP*, *GAPDH*, *POLR2e*, *RPLP0*, *SDHA* and *RPL13α* were determined as being the most suitable reference gene set to generate accurate relative gene expression data (**Figure 4**). Eight target genes were also detected in all samples and normalized to the above 6 reference genes using qBase+ software including a pure endogenous muscle control group that was fresh. The results were presented as the mean CNRQ values and analyzed using GraphPad Prism software. The results were mainly compared between the experimental group and the corresponding control group, the continuous application group and the single administration group and the changes at different time points.

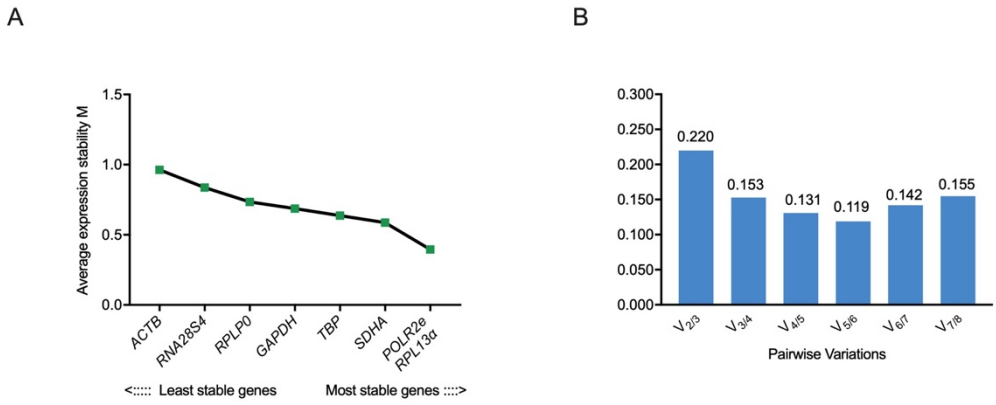

Figure 4. The determination of the optimal reference genes by GeNorm. (A) Genes ranked according to increasing expression stability, with the most stable genes (*POLR2e* and *RPL13α*) beginning from the right of the chart. (B). Results of the pairwise variation (V) between two sequential normalization factors containing an increasing number of genes revealing the optimal quantity of the reference gene. The lowest V value ($V_{5/6}$) was set as the cut-off value below which participation of more reference genes was redundant. Finally, *TBP*, *GAPDH*, *POLR2e*, *RPLP0*, *SDHA* and *RPL13α* were considered as the most suitable reference gene set for the project.

3.1.1 *ACAN*

ACAN was increased significantly in all treatment groups in relation to controls when morphogens and their different combination were applied continuously for the 7, 14, 30 days *in vitro* culturing period. On day 7, the relative expression level in the group treated with BMP-2 only showed a significantly greater expression than all other treatment modalities at that time point. By day 14, this trend was then replaced by the TGF-β_3 + OP-1 followed by the BMP-2 + TGF-β_3 + OP-1 group at day 30. There were no significant changes in the relative expression levels of *ACAN* in the control group over time. The relative expression levels in BMP-2 and BMP-2 + TGF-β_3 continuous treatment groups decreased sharply from day 7. *ACAN* peak values were found in TGF-β_3 alone, OP-1 alone, BMP-2 + OP-1 and TGF-β_3 + OP-1 groups on day 14 which remained then stable throughout the culture period. No significant change of *ACAN* was found in the BMP-2 + TGF-β_3 + OP-1 group from day 7 to 14, but only then there was an increase and the mean value exceeded other groups on day 30 (**Figure 5, Appendix A**).

At all the three time points, the relative expression levels of *ACAN* in continuous morphogen application groups were significantly higher than that in the corresponding treatment groups that had only been applied for 48h. Although there were differences in the expression patterns of *ACAN* when morphogens and their combinations were applied initially for 48h, none of the experimental groups were able to maintain the gene expression pattern as culture time increased to 30 days. No significant change was

found compared to the controls. The only notable difference was found in 48h OP-1, 48h BMP-2 + TGF-β_3 and 48h BMP-2 + OP-1 groups in which *ACAN* was significantly decreased by day 14, followed by a significant increase by day 30 that was almost equal to other treatment parameters (**Figure 5, Appendix B**).

3.1.2 *SOX9*

The relative expression levels of *SOX9* in nearly all experimental groups were stimulated continuously for 7, 14 and 30 days *in vitro* culturing period were significantly higher than that in the corresponding control group, except the BMP-2 and BMP-2 + TGF-β_3 + OP-1 groups on day 14. Similar to the *ACAN*, the highest relative expression level of *SOX9* were BMP-2, TGF-β_3 + OP-1 and BMP-2 + TGF-β_3 + OP-1 groups on day 7, 14, 30, respectively. The relative expression level in the BMP-2 continuous treatment groups dropped rapidly on day 14 and continued to decrease at a lower rate, which is similar to the time pattern in *ACAN* expression. TGF-β_3 alone and TGF-β_3 + OP-1 continuous treatment groups showed an upward trend from day 7 to 14 but without statistical significance. The other continuous stimulation experimental groups kept stable after reaching their peak on day 7 (**Figure 6, Appendix A**).

At all the three time points, the relative expression levels of *SOX9* in continuous morphogen application groups were significantly higher than in the corresponding treatment groups that had only been applied for 48h, except for the BMP-2 alone and BMP-2 + TGF-β_3 + OP-1 treated groups on day 14. The relative expression levels of

SOX9 on day 30 became negative in all single stimulation groups. 48h application of BMP-2 + TGF-β_3 resulted in the consistently and significantly lower relative expression levels than that in the corresponding control group on day 7 and 14. The single dose of OP-1 group showed significantly lower results in relation to the corresponding control group only on day 7, while TGF-β_3 + OP-1 group was lower on 14. The other single stimulation groups did not show any difference from the corresponding control group at three time points (**Figure 6, Appendix B**).

3.1.3 *Col2α1*

Col2α1 was increased significantly in all treatment groups in relation to controls when morphogens and their different combination were applied continuously for the 7 and 14 days *in vitro* culturing period, except the TGF-β_3 group on day 7. On day 7, the relative expression level in the group treated with BMP-2 only showed a significantly greater expression than all other treatment modalities at that time point which was similar to the results of *ACAN* and *Sox9*, but this trend was then replaced by the TGF-β_3 + OP-1 group on day 14. On day 30, the mRNA of *Col2α1* could not be detected in some samples received the continuous stimulations using qRT-PCR, resulting in the data volume of TGF-β_3 and BMP-2 + OP-1 groups could not meet the requirements of the statistical analysis. Most of the groups stimulated continuously showed a decreasing or unchanged trend over time from day 7, but BMP-2 + TGF-β_3 group reached the peak on day 14. Additionally, the relative expression levels of *Col2α1* in the group applied BMP-2 continuously decline significantly after day 7, which was similar to the time

pattern of *ACAN* and *SOX9* (**Figure 7, Appendix A**).

The mRNA of *Col2α1* could be detected in all groups applied the proteins for 48h, alone or in varying combinations. At all the three time points, the relative expression levels of *Col2α1* in continuous morphogen application groups were significantly higher than that in the corresponding treatment groups that had only been applied for 48h. The results were all negative in groups applied once on day 14. On day 30, the relative expression levels in groups applied BMP-2 + OP-1 and BMP-2 + TGF-β_3 + OP-1for 48h were up-regulated and significantly higher than that in the corresponding control group (**Figure 7, Appendix B**).

3.1.4 *Col1α1*

At all the three time points, the relative expression levels of *Col1α1* maintained positive only in control group and the group applied TGF-β_3 for 48h. Except the above two groups, the relative expression levels of *Col1α1* in the other groups reached their bottom on day 7, no matter they received a single or continuous stimulation. Thereafter, all groups presented an upward trend and reached the highest value on day 30 and became positive except the group applied BMP-2 + OP-1 continuously. After continuous stimulation, the relative expression levels of *Col1α1* in most experimental groups were significantly lower than that in the corresponding control group, except the OP-1 group on day14, TGF-β_3 + OP-1 and BMP-2 + TGF-β_3 + OP-1 groups on day 30 showed no difference and TGF-β_3 group on day 30 had higher expression. At all the three time

points, the relative expression levels of *Col1α1* in continuous morphogen application groups were significantly lower than that in the corresponding treatment groups that had only been applied for 48h, which was contrary to the results of *ACAN* and *SOX9* (**Figure 8, Appendix A and B**).

3.1.5 *Col10α1*

Although the total mRNA was extracted from six samples in each experimental group stimulated continuously, but the expression of *Col10α1* could not detected resulting that the data size did not meet the statistical requirements for analyses. Therefore, more information about *Col10α1* could not be obtained in groups which were applied the growth factors continuously.

The relative expression of *Col10α1* also could not be detected in the groups applied TGF-β_3 + OP-1 or BMP-2 + TGF-β_3 + OP-1 for 48h on day 7; however, it became measurable in these two groups on day 14 and 30. Nevertheless, all available data obtained from the single stimulation groups was negative at any time point, except the BMP-2 + TGF-β_3 + OP-1 group on day 30. There was no difference in other valid data when compared with the corresponding control group at all three time points, except the group applied OP-1 once on day 7 and the group applied BMP-2 + OP-1 once on day 30 (**Figure 9, Appendix B**).

3.1.6 *ALP*

The relative expression level of *ALP* in group applied BMP-2 alone continuously on day 7 was the only positive data in all groups and at all time points. The other groups stimulated continuously showed a decreasing trend from the beginning of the detection time point. On day 7 and 14, the relative expression level of *ALP* in groups applied continuously were higher than that in corresponding groups stimulated for 48h, but the results were reversed in all the experimental groups with BMP-2 participation on day 30 (**Figure 10, Appendix A and B**).

3.1.7 *Col1α1*, *Col2α1* and *ALP*

The relative expression levels of *Col1α1*, *Col2α1* and *ALP* in the control group and the experimental groups applied the same morphogens continuously for 7, 14 and 30 days were compared base on the time point. In the control group, the order of relative expression levels of these three genes from high to low is *Col1α1*, *Col2α1* and *ALP* at all time points. *Col2α1* was expressed significantly stronger than the other two genes in all experimental group on day 7 and 14; however, the difference between *Col1α1* and *Col2α1* in the groups applied BMP-2 alone, BMP-2 + TGF-β_3 and BMP-2 + TGF-β_3 + OP-1 continuously became insignificant on day 30. The relative expression levels of *Col2α1* maintained dominant from day 7 to 30 in groups applied OP-1 and TGF-β_3 + OP-1 continuously, while the *Col1α1* became the greatest one in the other groups on day 30. The relative expression level of *ALP* shared the similar time pattern with *Col2α1* in the group applied BMP-2 alone continuously, while in the other groups it maintained

negative and keep the lowest relative expression level at most time points (**Figure 11**).

3.1.8 *VEGF-A*

Most experimental groups stimulated continuously showed the significantly higher relative expression level of *VEGF-A* than that in the corresponding control group on day 7, expect the group applied BMP-2 alone continuously. By day 14, only the groups stimulated by TGF-β_3 alone, BMP-2 + TGF-β_3 and BMP-2 + TGF-β_3 + OP-1 continuously were found the significantly higher relative expression levels than that of the control. The values in all experimental groups on day 30 were either not different from that of the control group or significantly lower (**Figure 12, Appendix A**).

At all the three time points, the relative expression levels of *VEGF-A* in continuous morphogen application groups were significantly higher than that in the corresponding treatment groups that had only been applied for 48h, except for the BMP-2 alone treated groups on day 7. The relative expression levels of *VEGF-A* in all experimental groups stimulated for 48h showed no difference with the control group on day 7. By day 14, among all the single dose experimental groups, only the groups applied OP-1 and TGF-β_3 + OP-1 showed positive value though lower than that in the corresponding control group (**Figure 12, Appendix B**).

3.1.9 *Col4α1*

The relative expression levels of *Col4α1* in the control group decreased continuously and became negative on day 30, while the value of the other continuous stimulation groups maintained positive during the entire culture. The values in most experimental groups applied morphogens continuously showed a downward trend from day 7, except OP-1 alone treated group decreased from day 14 and BMP-2 + TGF-β_3 treated group increased significantly after reaching the bottom on day 14. The highest relative expression level of *Col4α1* on day 7 and 14 was found in the group applied BMP-2 + TGF-β_3 + OP-1 continuously (**Figure 13, Appendix A**).

The relative expression levels of *Col4α1* in continuous morphogen application groups on day 7 were higher than that in the corresponding treatment groups that had only been applied for 48h and significant difference was found in most experimental groups except the TGF-β_3 alone and OP-1 alone treated groups (**Figure 13, Appendix B**).

3.2 Histomorphometric assessment

3.2.1 Alcian Blue staining

Alcian blue would show the presence of glycosaminoglycans in cartilages and other body structures and the positive area was stained in blue. The positive area was found in nearly all the groups at all time points, no matter the control group or experimental groups stimulated continuously or only for 48h. The positive areas were mainly in the

intercellular space of muscle cells or near the fascia (**Figure 14**).

According to the semi-quantitative analysis of alcian blue staining, the positive area ratios were lower than 5% at three time points, although it presented an increasing trend. The positive area ratio in the experimental groups stimulated continuously significantly higher than that in the corresponding control group at all time points, except BMP-2 + TGF-β_3 and BMP-2 + TGF-β_3 + OP-1 treated groups on day 7 and TGF-β_3 treated group on day 14. The highest positive area ratio was found in the group treated BMP-2 + OP-1 continuously on day 14. The comparison between the experimental groups applied the same morphogens continuously and for only 48h showed the significant difference in BMP-2 + OP-1 treated groups on day 7 and most experimental groups on day 14 and 30 (**Figure 15**).

3.2.2 Aggrecan Immunohistochemistry

By means of IHC, in which aggrecan was treated as the antigen, the green area indicated the positive antigen-antibody interactions. The positive area was detected in nearly all the groups at all time points, no matter the control group or experimental groups stimulated continuously or only for 48h, although the positive reaction was very weak in the control group and the single stimulation experimental groups. The positive areas appeared in any part of the sections, including the muscle cells, the intercellular space and the fascia (**Figure 14**).

The semi-quantitative analysis of the IHC showed the MOD in all continuous

application groups were significantly higher than that in the corresponding groups stimulated for 48h at all time points. The group applied BMP-2 continuously showed the highest MOD among all groups on day 14. Similar to the time pattern of the relative expression levels of chondrogenesis-related genes in 7 to 14 days, the MOD value of in the group applied BMP-2 continuously declined sharply after reaching the peak on day 14. Additionally, the strongest positive reaction on day 30 was found in the BMP-2 + TGF-β_3 continuous group, although there was not significantly different from the group applied TGF-β_3 + OP-1 continuously (**Figure 16**).

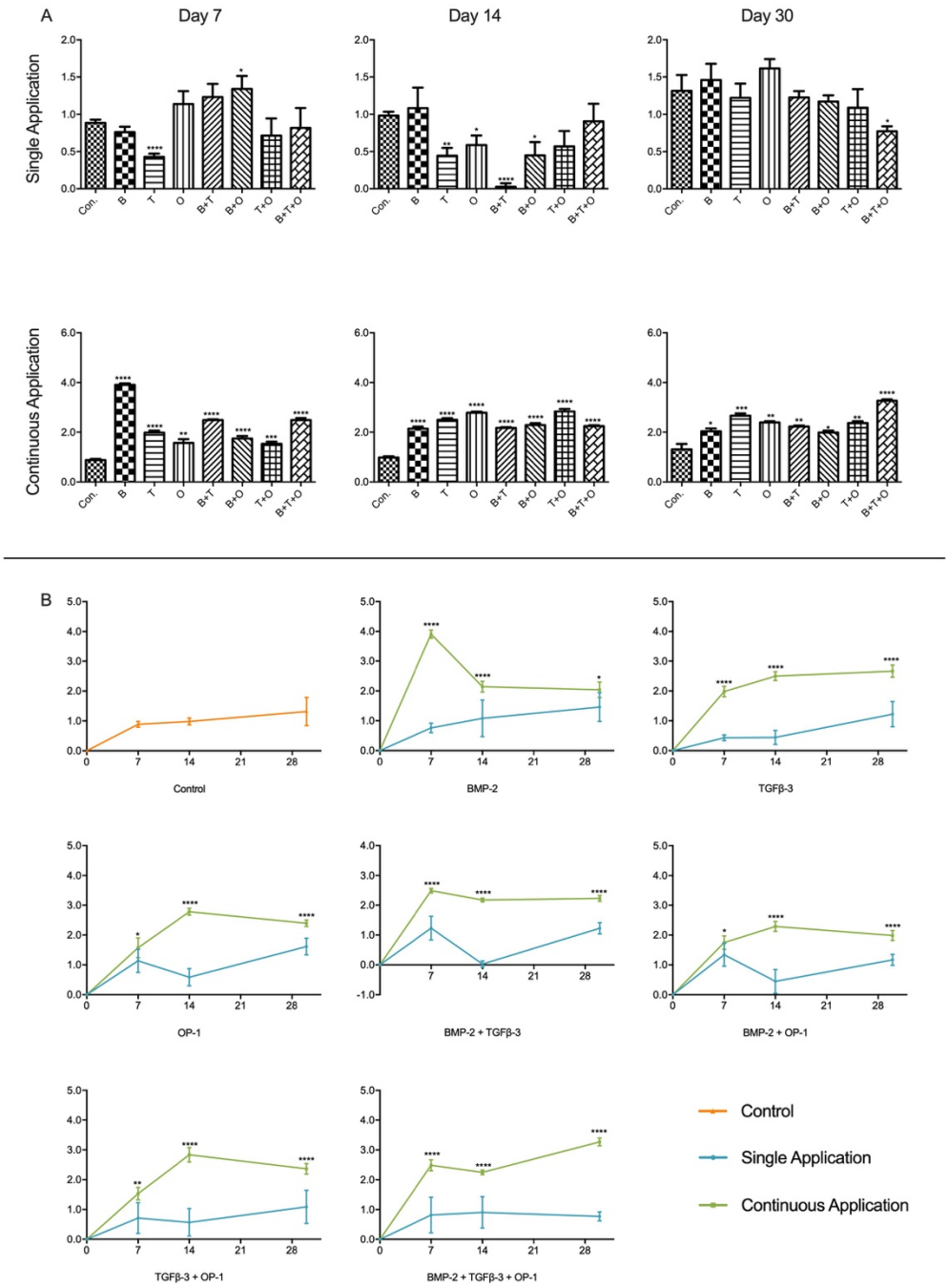

Figure 5. The analyses of the relative expression levels of *aggrecan* (*ACAN*). The results were presented as calibrated normalized relative quantity (CNRQ). (A) Comparisons between each experimental group under different stimulation modes and the corresponding control group on day 7, 14 and 30 using one-way ANOVA. (B) Comparisons between the experimental groups with single and continuous application of the same combination of growth factors on day 7, 14 and 30 using two-way ANOVA. Con.= control group, B= BMP-2 treated group, T= TGF-β3 treated group, O= OP-1 treated group, B+T = BMP-2 + TGF-β_3 treated group, B+O=BMP-2 + OP-1 treated group, T+O= TGF-β_3 + OP-1 treated group, B+T+O= BMP-2 + TGF-β_3 + OP-1 treated group. We defined $p<0.05$ as a statistically significant difference. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

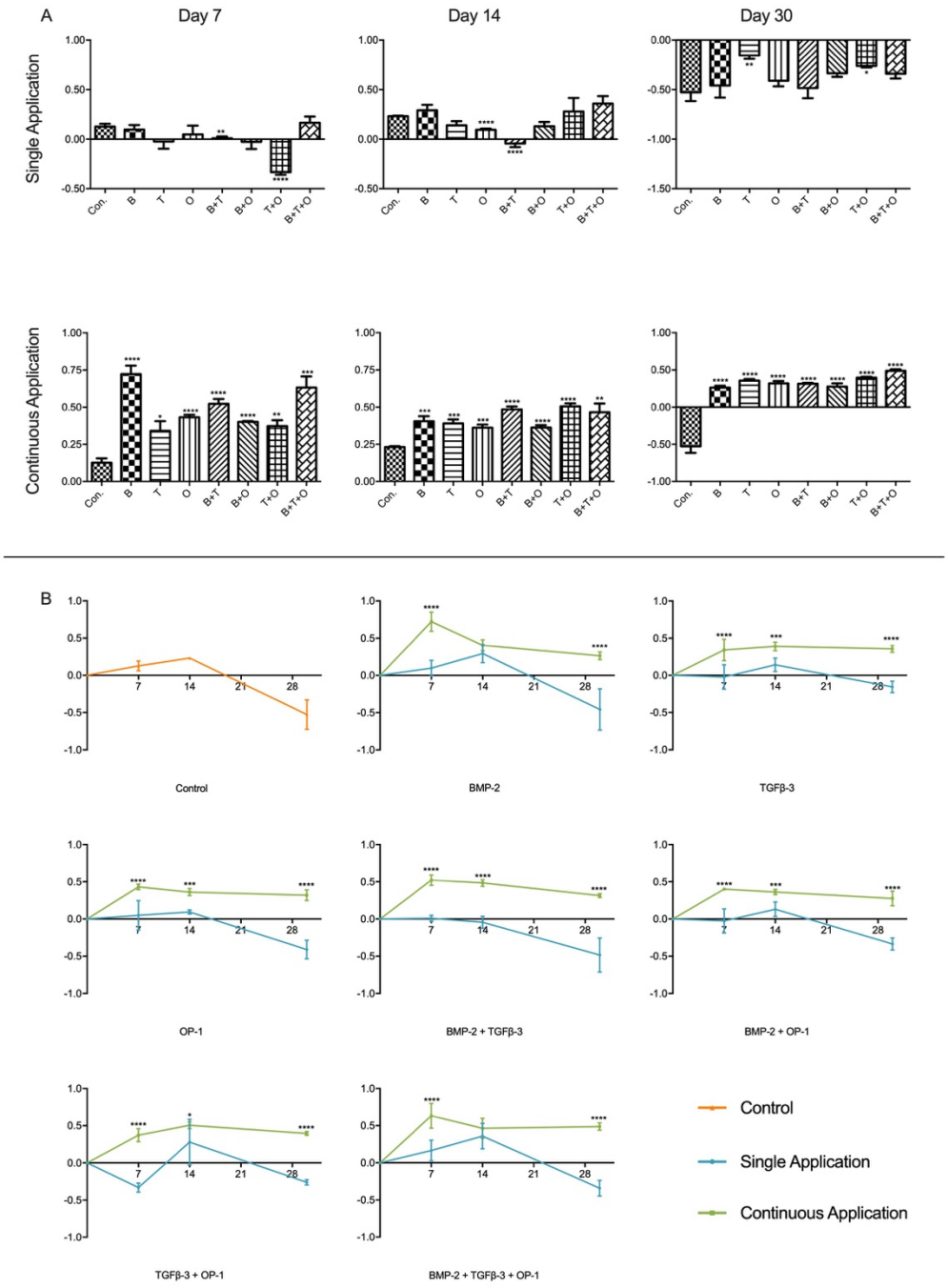

Figure 6. The analyses of the relative expression levels of *SRY (Sex Determining Region Y)-Box 9* (*SOX9*). The results were presented as calibrated normalized relative quantity (CNRQ). (A) Comparisons between each experimental group under different stimulation modes and the corresponding control group on day 7, 14 and 30 using one-way ANOVA. (B) Comparisons between the experimental groups with single and continuous application of the same combination of growth factors on day 7, 14 and 30 using two-way ANOVA. We defined $p<0.05$ as a statistically significant difference. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

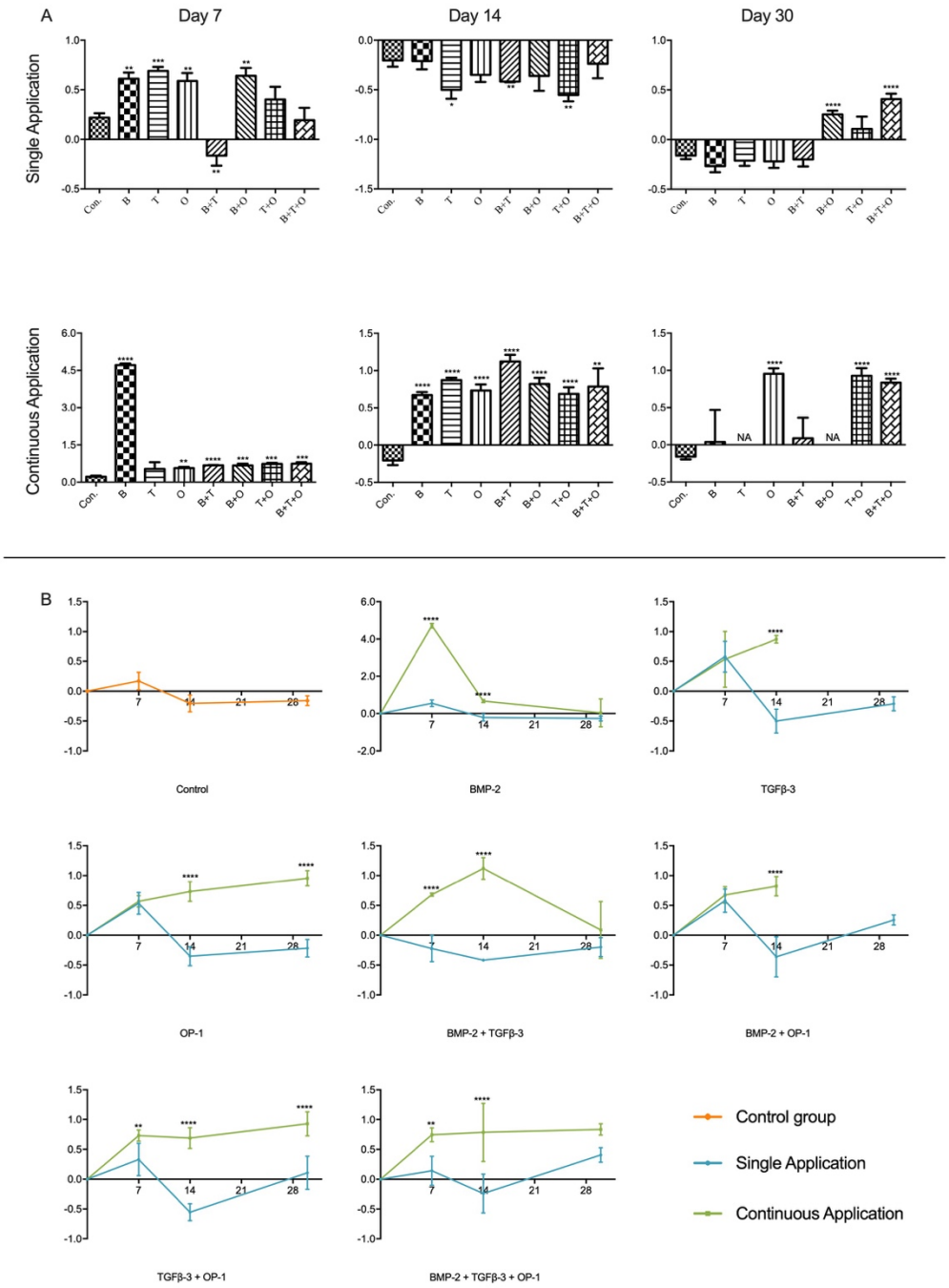

Figure 7. The analyses of the relative expression levels of *collagen type II alpha 1* (*Col2α1*). The results were presented as calibrated normalized relative quantity (CNRQ). (A) Comparisons between each experimental group under different stimulation modes and the corresponding control group on day 7, 14 and 30 using one-way ANOVA. (B) Comparisons between the experimental groups with single and continuous application of the same combination of growth factors on day 7, 14 and 30 using two-way ANOVA. The data volume in the experimental groups applied TGF-β_3 and BMP-2 + OP-1 continuously on day 30 could not meet the requirements of the statistical analysis. NA= not available. We defined $p<0.05$ as a statistically significant difference. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

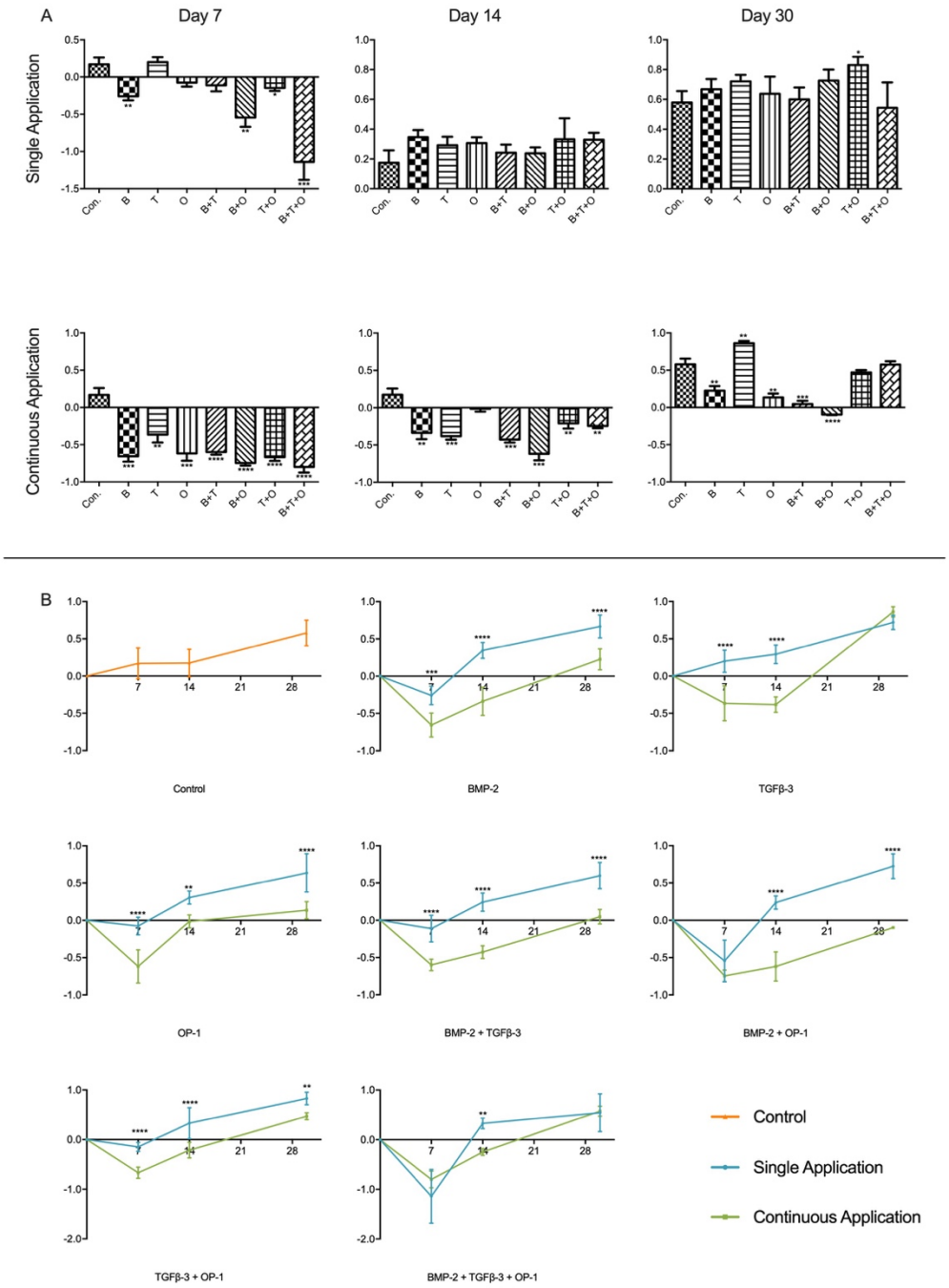

Figure 8. The analyses of the relative expression levels of *collagen type I alpha 1* (*Col1α1*). The results were presented as calibrated normalized relative quantity (CNRQ). (A) Comparisons between each experimental group under different stimulation modes and the corresponding control group on day 7, 14 and 30 using one-way ANOVA. (B) Comparisons between the experimental groups with single and continuous application of the same combination of growth factors on day 7, 14 and 30 using two-way ANOVA. We defined $p<0.05$ as a statistically significant difference. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

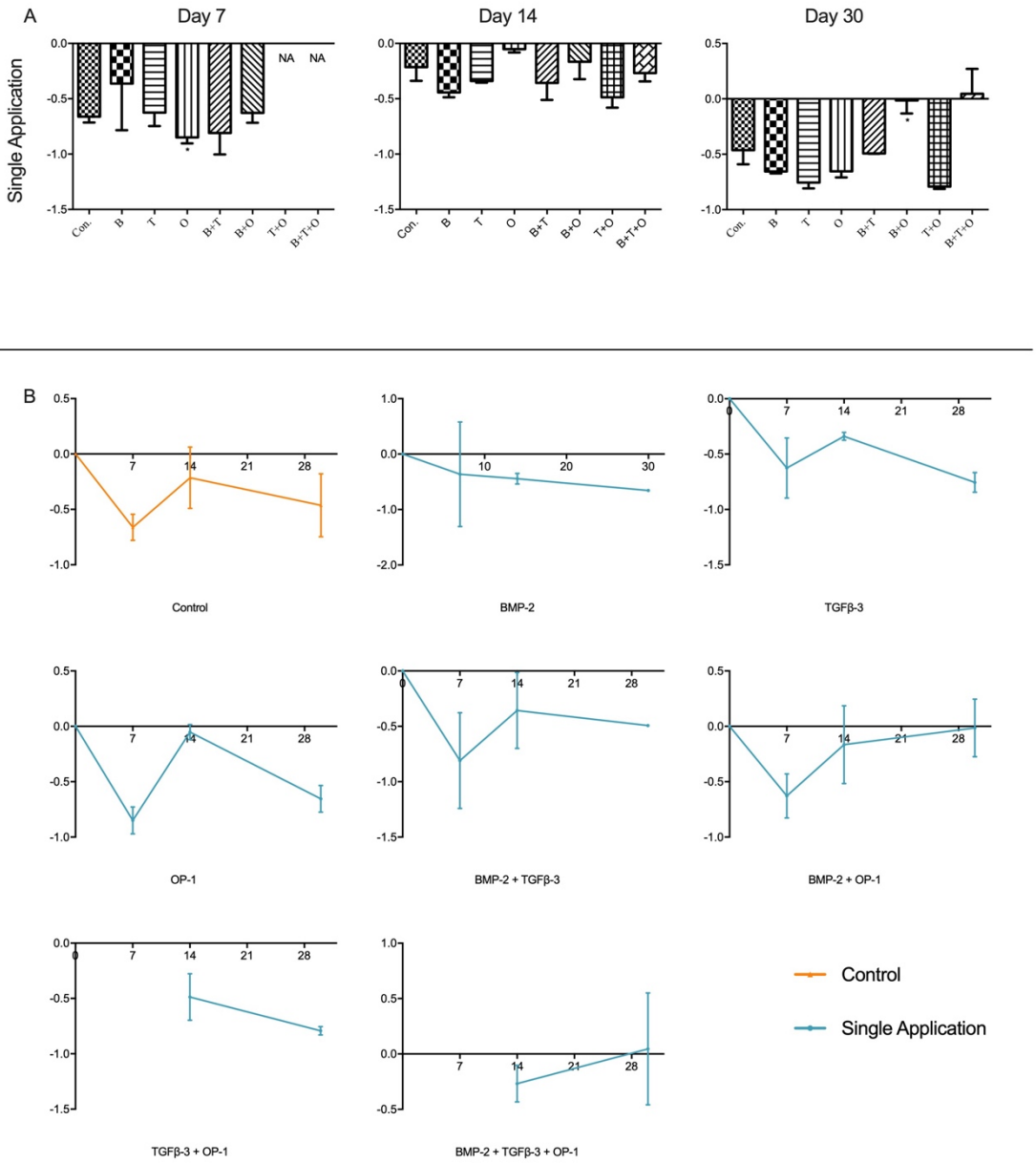

Figure 9. The analyses of the relative expression levels of *collagen type X alpha 1* (*Col10α1*). The results were presented as calibrated normalized relative quantity (CNRQ). (A) Comparisons between each experimental group under a single stimulation and the corresponding control group on day 7, 14 and 30 using one-way ANOVA. (B) Changes in the relative expression levels of *Col10α1* in the experimental groups with a single application of the same combination of growth factors on day 7, 14 and 30 using one-way ANOVA. The data volume in all the experimental groups stimulated continuously could not meet the requirements of the statistical analysis. The data volume in the experimental groups applied TGF-β_3 + OP-1 and BMP-2 + TGF-β_3 + OP-1 for 48h could not meet the requirements of the statistical analysis on day 7. NA= not available. We defined $p<0.05$ as a statistically significant difference. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

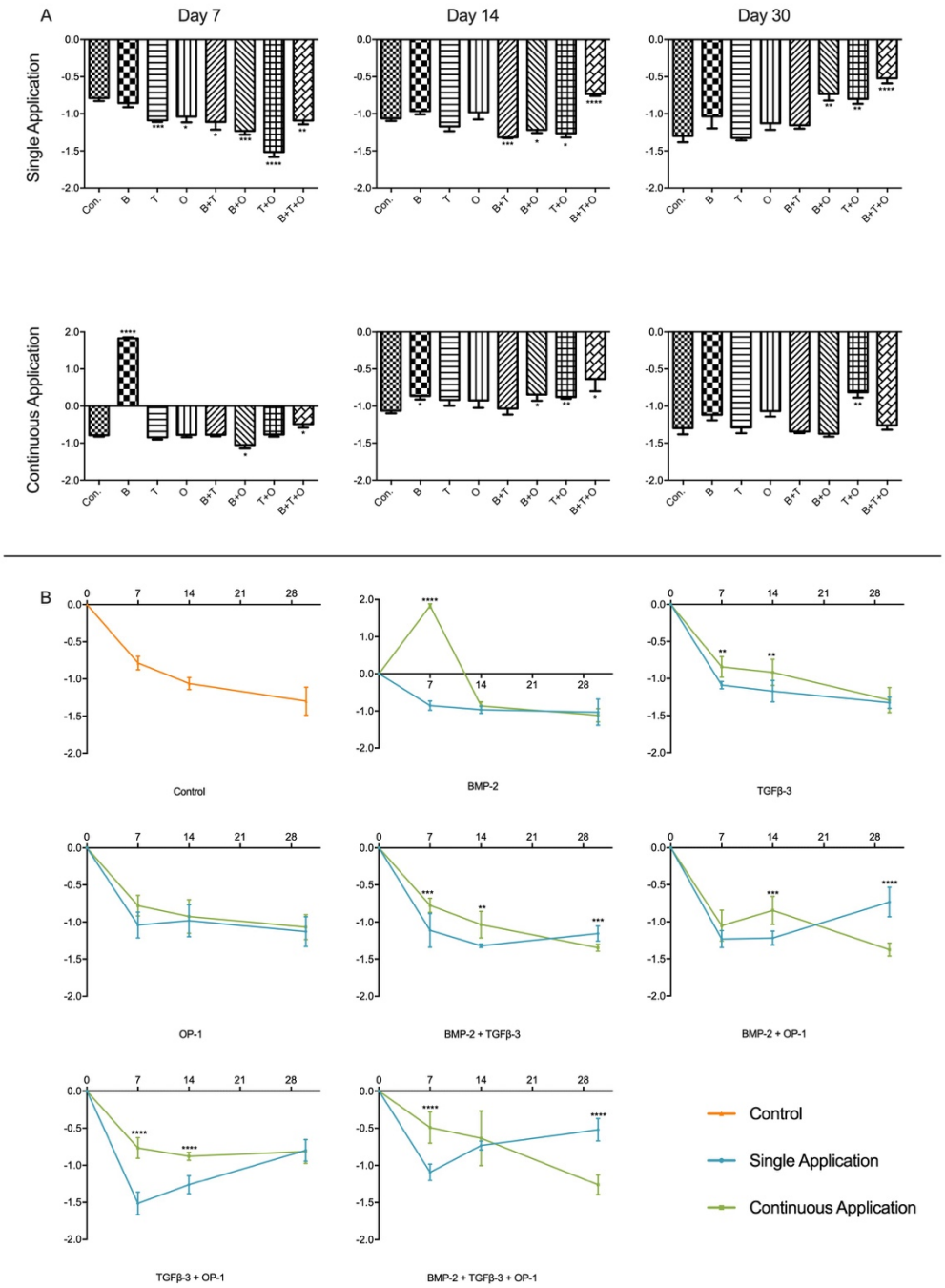

Figure 10. The analyses of the relative expression levels of *alkaline phosphatase* (*ALP*). The results were presented as calibrated normalized relative quantity (CNRQ). (A) Comparisons between each experimental group under different stimulation modes and the corresponding control group on day 7, 14 and 30 using one-way ANOVA. (B) Comparisons between the experimental groups with single and continuous application of the same combination of growth factors on day 7, 14 and 30 using two-way ANOVA. We defined $p<0.05$ as a statistically significant difference. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

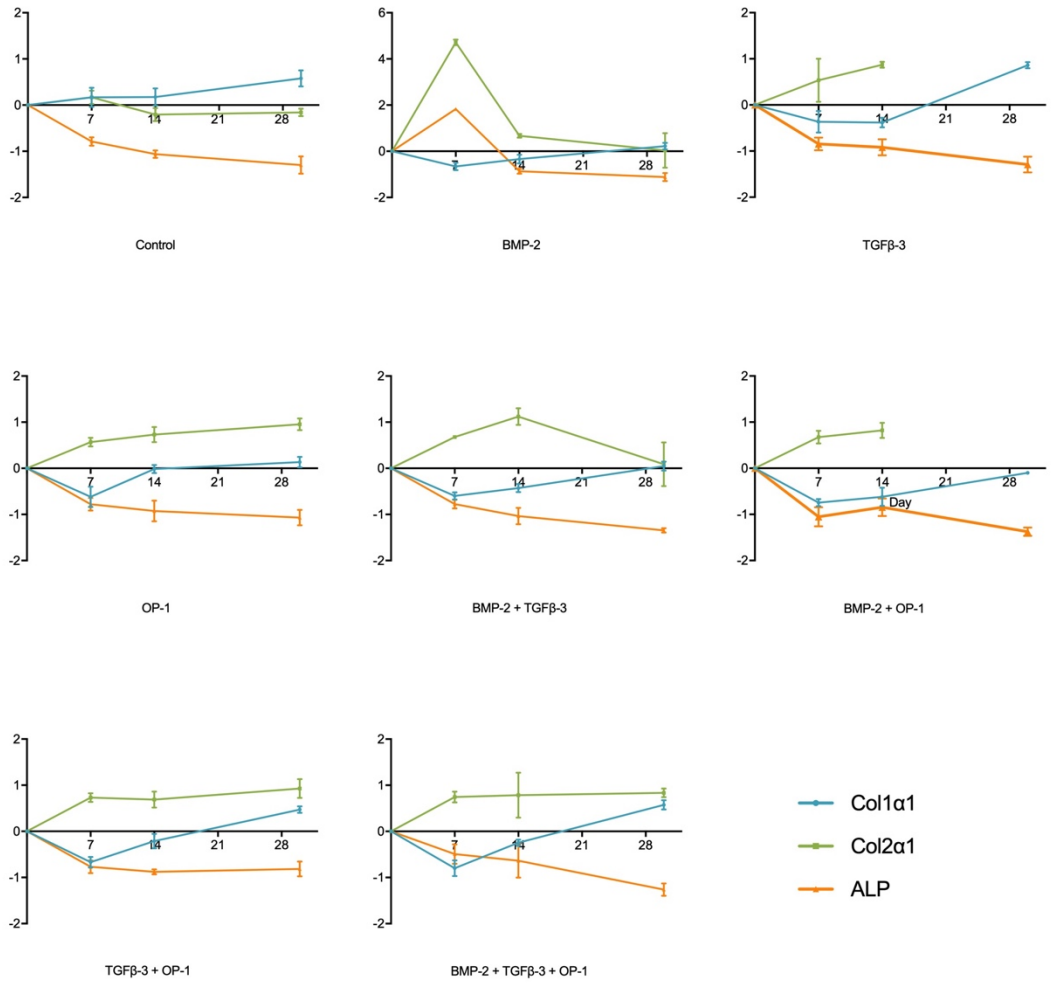

Figure 11. The analyses of the relative expression levels of *Col1α1*, *Col2α1* and *ALP* in the group applied the same morphogens continuously at all the time points. The data volume of *Col2α1* in TGF-β_3 and BMP-2 + OP-1 group on day 30 could not meet the requirements of the statistical analysis. B= BMP-2 treated group, T= TGF-β3 treated group, O= OP-1 treated group, B+T = BMP-2 + TGF-β_3 treated group, B+O=BMP-2 + OP-1 treated group, T+O= TGF-β_3 + OP-1 treated group, B+T+O= BMP-2 + TGF-β_3 + OP-1 treated group.

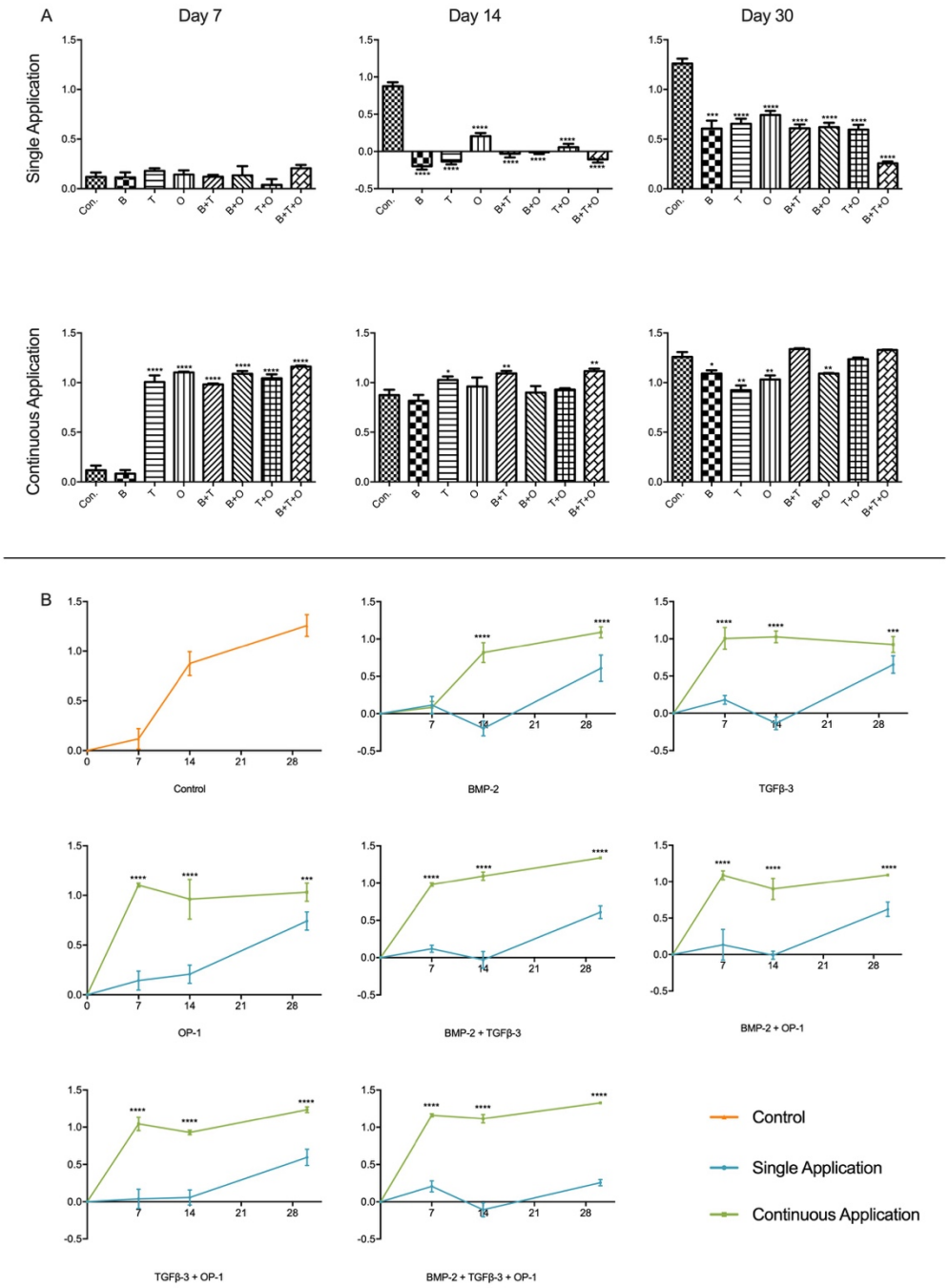

Figure 12. The analyses of the relative expression levels of *vascular endothelial growth factor A* (*VEGF-A*). The results were presented as calibrated normalized relative quantity (CNRQ). (A) Comparisons between each experimental group under different stimulation modes and the corresponding control group on day 7, 14 and 30 using one-way ANOVA. (B) Comparisons between the experimental groups with single and continuous application of the same combination of growth factors on day 7, 14 and 30 using two-way ANOVA. We defined $p<0.05$ as a statistically significant difference. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

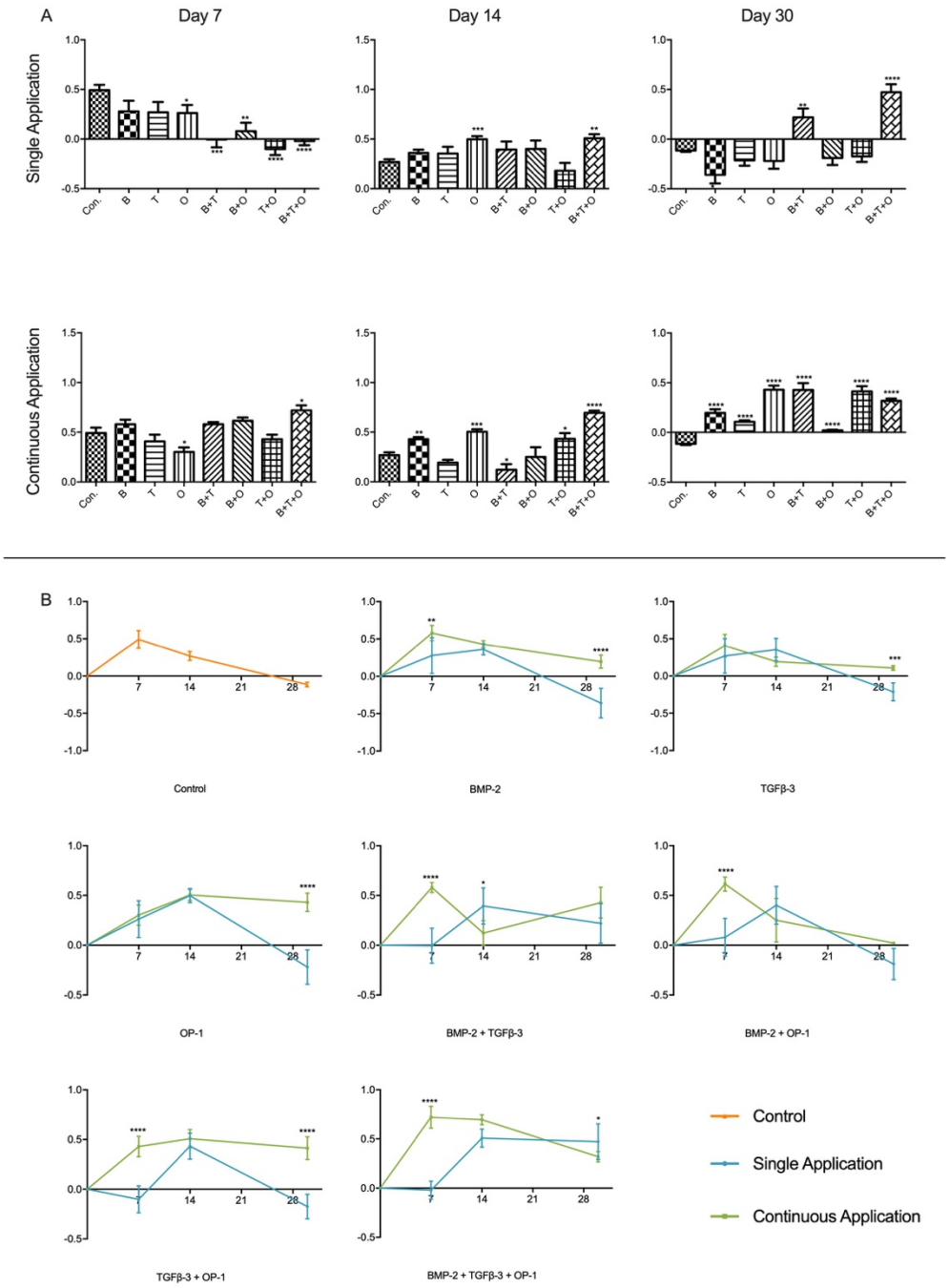

Figure 13. The analyses of the relative expression levels of *collagen type IV alpha 1* (*Col4α1*). The results were presented as calibrated normalized relative quantity (CNRQ). (A) Comparisons between each experimental group under different stimulation modes and the corresponding control group on day 7, 14 and 30 using one-way ANOVA. (B) Comparisons between the experimental groups with single and continuous application of the same combination of growth factors on day 7, 14 and 30 using two-way ANOVA. We defined $p<0.05$ as a statistically significant difference. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

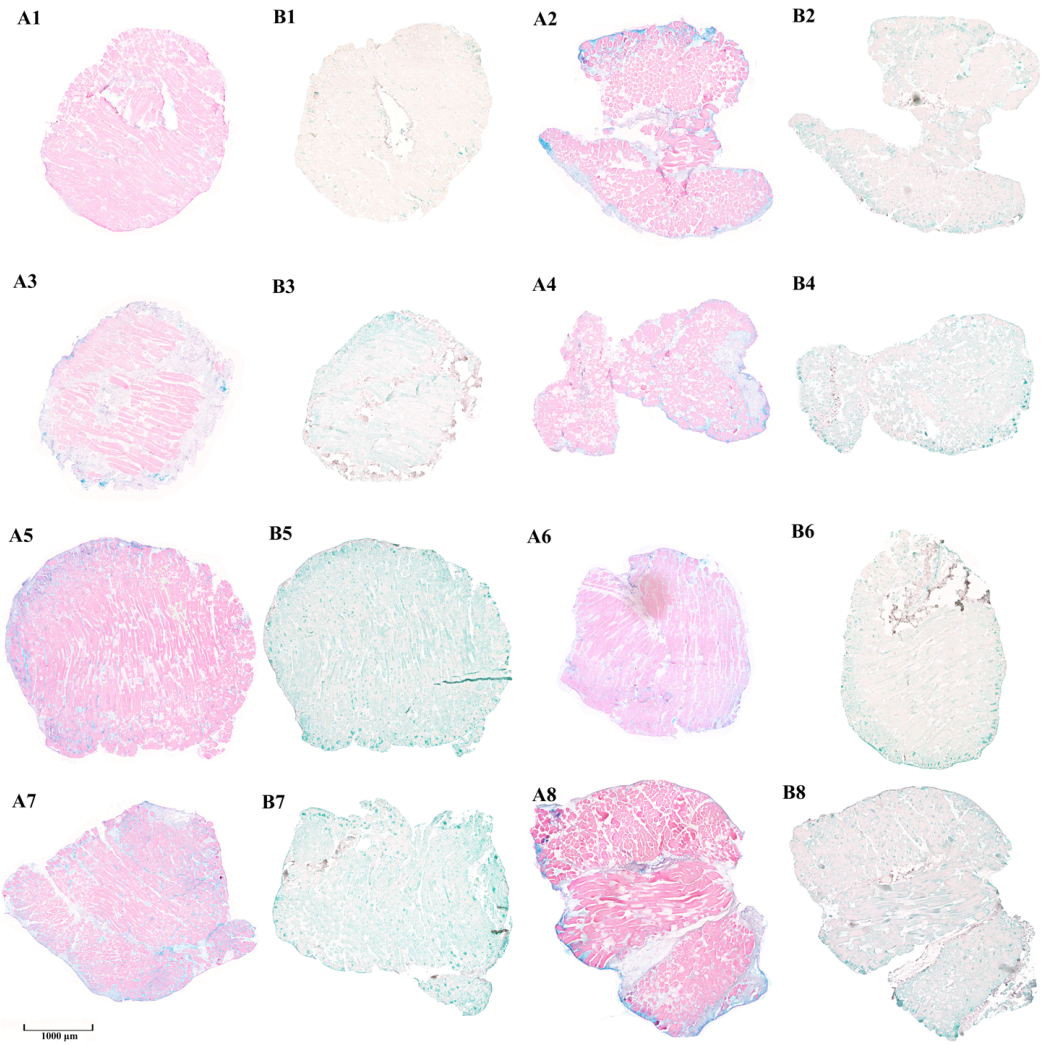

Figure 14. The staining results of the control group and the experimental groups stimulated continuously on day 30, including alcian blue staining and immunohistochemistry (IHC). (A1-A8). These figures represented the alcian blue staining results of control group and the experimental groups applied BMP-2 alone, TGF-β_3 alone, OP-1 alone, BMP-2 + TGF-β_3, BMP-2 + OP-1, TGF-β_3 + OP-1 and BMP-2 + TGF-β_3 + OP-1continuously, respectively. The color of positive reaction in alcian blue was blue which indicated the deposition of acidic polysaccharides. (B1-B8). These figures represented the aggrecan IHC results of control group and the experimental groups applied BMP-2 alone, TGF-β_3 alone, OP-1 alone, BMP-2 + TGF-β_3, BMP-2 + OP-1, TGF-β_3 + OP-1 and BMP-2 + TGF-β_3 + OP-1continuously, respectively. The target antigen in IHC was aggrecan. The color of the positive antigen–antibody interactions in this IHC was green.

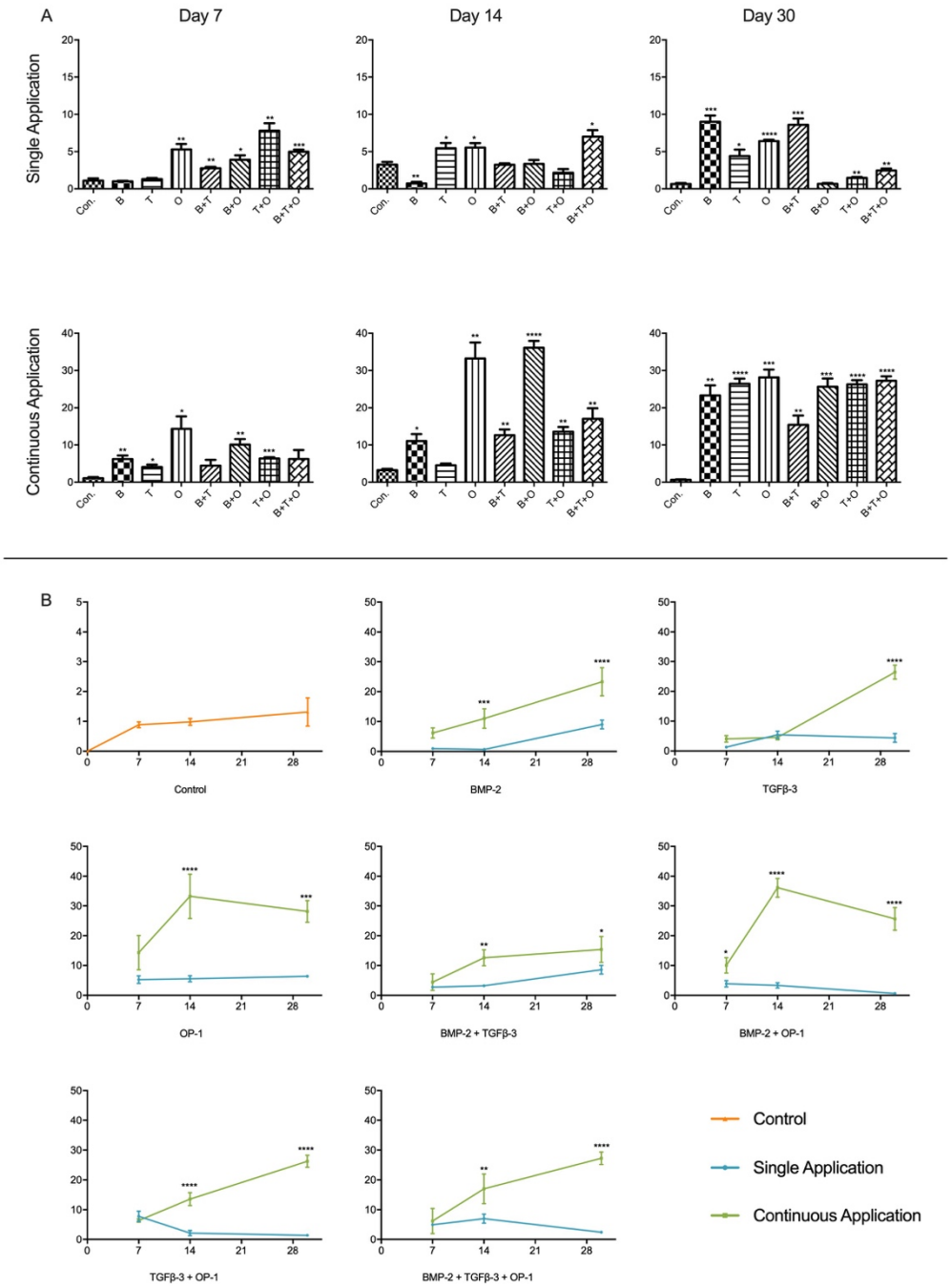

Figure 15. The semi-quantitative analysis of alcian blue staining. The results were presented as positive area ratio (%). (A) Comparisons between each experimental group under different stimulation modes and the corresponding control group on day 7, 14 and 30 using one-way ANOVA. (B) Comparisons between the experimental groups with single and continuous application of the same combination of growth factors on day 7, 14 and 30 using two-way ANOVA. We defined $p<0.05$ as a statistically significant difference. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

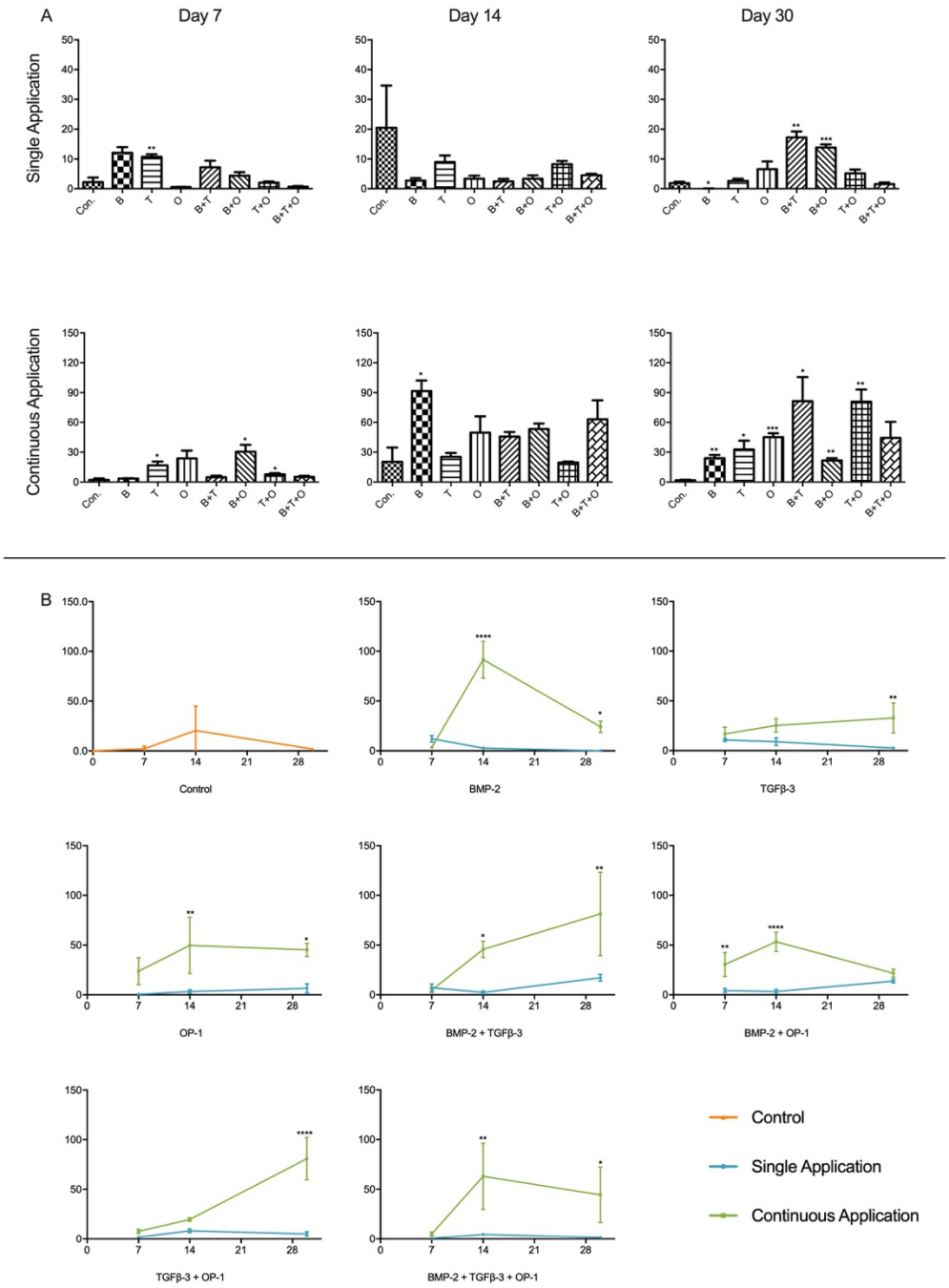

Figure 16. The semi-quantitative analysis results of immunohistochemistry (IHC). The results were presented as optical density value (MOD). The target antigen in IHC was aggrecan. (A) Comparisons between each experimental group under different stimulation modes and the corresponding control group on day 7, 14 and 30 using one-way ANOVA. (B) Comparisons between the experimental groups with single and continuous application of the same combination of growth factors on day 7, 14 and 30 using two-way ANOVA. We defined $p<0.05$ as a statistically significant difference. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

4. Discussion

4.1 The muscle tissue model

The study of morphogens/proteins/ligands and their effect on biological systems has historically always been performed on isolated stem or differentiated cells *in vitro*[1,138-140]. Cicione et al.[141], for example, investigated the capacity of BMP-2, OP-1, and TGF-β_3 and their chondrogenic differentiation effect on MSCs *in vitro* under normoxic conditions, whilst Gozo et al.[142] attempted to represent the Forkhead box protein C2-mediated regeneration and osteogenesis in muscle tissue *in vitro* by utilizing C2C12 myoblasts. Whilst cell-based research has shed much light on the signal transduction cascade and how morphogens affect the cell differentiation behavior, how accurate these interpretations are in relation to a tissue, which actually makes up an organism, remains questionable. Tissues are not entirely composed of a single cell type but a combination of different cells interacting with each other to produce a specific tissue wide response. If now an extracellular signal is presented, single cell response studies can only provide a basic single response yet do not reflect the response of the complexities of tissue since many types of cells and the cell-specific extracellular matrices are involved generating a cascading and often varying response[123,143,144]. The benefits of using tissue in research and for regenerative procedures remains far more beneficial than single stem cells. Studies have shown that stem cells containing in the muscle tissue can differentiate into several lineages *in vitro* and *in vivo*[125,126]. Moreover, it was shown that cells cultured *in vitro* after isolation from their natural environment could be disrupted from their homeostasis as critical essential amino acid building

blocks were lacking[145,146], while tissue *in vitro* cultures might release the critical components such as extra glucose and proteins[147-149], to assists in establishing a new homeostasis. Furthermore, the realization of tissue based regeneration will have clinical significance, which was to solve the time consuming and the expensive procedure caused by autologous cell transplantation[116,117].

For these apparent reasons, the present study thus chose to utilize a tissue based system, here a muscle tissue based model, to provide familiar internal milieu as that of an *in vivo* system together with a broader spectrum of tissue based response to the application of different morphogens and their combinations. Although it was difficult to observe tissue viability under a microscope, like in cell cultures, the IHC and qRT-PCR results confirmed that tissue can be made to survive *in vitro* and reflect accurately the process of morphogenesis as it might occur *in vivo*. The relative expressions of *Col4α1*, a biomarker for angiogenesis[133,150-152], and the expression of *VEGF-A*, a marker for development and proliferation of endothelial cells[153], were all up-regulated in all treatment groups throughout the 30 days *in vitro* culture period. Whilst the expressional level has to be carefully considered to *in vivo* based system, the fact that these two markers were increased confirmed that muscle tissue for use in assessing morphogen based related effects is an effective model that provides an accurate prediction of how the tissue would respond *in vivo*.

4.2 Chondrogenesis: articular vs. non-articular

The results of IHC and alcian blue staining showed that morphogenesis and specific ECM can be induced in a culture system by applying growth factors, on their own or in different combinations. ACAN, as in the IHC detection, is one of the abundant proteoglycans in the all types of cartilage as it is the fundamental for cartilage function and skeletal development[154,155]. Alcian blue first used by Steedman et al.[156] as a selective dye for mucins in 1950 and then applied to stain acidic polysaccharides such as glycosaminoglycans in cartilages and other body structures[157] was used in the study in conjunction with the IHC results to validate that chondrogenesis did indeed occur *in vitro.* In previous studies, Yoon and Lyons[158] described that *SOX9* was continuously expressed in chondrocytes up to the hypertrophic stage and was also involved in the BMPs induced chondrogenesis, which was consistent with our results of qRT-PCR. *SOX9* served as the indicator for the initial activation of general chondrogenesis and regulated[12,159,160] the transcriptional program by up-regulating *Col2α1* and *ACAN*[161-163]. The significantly higher expression of *ACAN* and *SOX9* genes in the experimental groups indicated the trend of chondrogenic morphogenesis.

The polymeric extracellular framework is composed of collagen in almost all animal tissue[164,165]. The collagen type I, collagen type X and elastin are the unique components in fibrocartilage, hypertrophic cartilage and elastic cartilage, respectively, while the predominant compositions of hyaline cartilage are water (80%) and collagen type II (10%)[166-169]. The relative expression levels of *Col1α1*, *Col2α1* and *Col10α1* were all

assessed by qRT-PCR in this study. The absence of *Col10α1* in nearly all experimental group under continuous stimulation suggested that the chosen three growth factors, BMP-2, OP-1 and TGF-β_3, on their own or combinations could be inhibiting chondrocyte hypertrophy or endochondral ossification processes. Similarly, Gonzalez-Fernandez et al.[170] found that hypertrophy could be suppressed and more stable chondrogenesis be produced when TGF-β_3 and BMP-2 were co-delivered with MSCs. Whilst, Cals et al. [171] did not find any significant TGF-β dependent differences along with the expression of *Col10α1* other studies have also demonstrated that *Col10α1* expression increased during the chondrogenesis of MSCs without any BMPs[172,173].

Articular cartilage matrix is composed of 90-95% collagen type II and is the most sought after molecule during the tissue engineering of this matrix. Any other collagen or low levels of collagen type II content have been a thorn in the side of articular cartilage based therapies as most treatment revert to fibrocartilage or ossify completely[174,175]. The relative expression level of *Col2α1* was significantly increased in all treatment groups in relation to controls when morphogens and their different combination were applied continuously for the 7 and 14 days *in vitro* culturing period, except the TGF-β_3 group on day 7. This promising outcome would mean the muscle tissue was transforming itself towards a possible articular cartilage lineage, critical for tissue engineering prospect in healing arthritic defects. The negative CNRQ values of *Col1α1* on day 7 and 14 irrespective of the type of morphogen used in the present study or their varying combination eliminated also the aspect of tissue transformation towards

a fibrocartilage lineage. Hence, the results of the comparison between *Col1α1* and *Col2α1* on day 7 and 14 showed that continuous application of the chosen morphogens from the TGF-β supergene family of proteins had the capability of forming hyaline cartilage. While Wang et al.[169] revealed that chondrocyte predominantly expressed collagen type I at the initial stage (1-3 days), even exceeding collagen type II, according to the time-dependent detections of cartilage-specific ECM protein *in vitro*, our study suggested otherwise perhaps as we used tissue and not cells and the lack of an early detection.

However, whilst the initial findings at day 7 and 14 were indicative of a hyaline articular cartilage formation process, the observation in the recovery of *Colα1* on day 30 in most experimental groups, suggested that fibrosis was in progress. One possible explanation was that the damaged cartilage was replaced by fibrocartilage consisting with a high amount of collagen type I[176]. Alternatively, other studies also showed that the ossification, which also relates to the expression of *Colα1* may occur after 30 days, *in vivo* [133,177], strongly suggesting that 30 days as a terminal detection time point may be too early to make conclusions as there patterns of expression could deviate as culture time increases.. Long culture studies are necessary to eliminate this concern. Therefore, whether the relative expression of *Col1α1* peaked before day 7, after day 30 or both, was unclear due to the limitation of detection time. Moreover, most of the experimental groups in which OP-1 was used maintained a significantly higher expression level of *Col2α1*, suggesting that OP-1 may be a key factor in maintaining the formation of

hyaline articular cartilage. However, the absence of the qRT-PCR results in some experimental group when amplifying *Col2α1* made the analysis incomplete, which may have been caused by the destabilization during the translation of some mRNA with AU-rich elements[178-180].

4.3 The "withdrawal effect"

The MOD values, reflecting the density of aggrecan, in most groups stimulated continuously for 14 and 30 days were significantly higher than that in the corresponding single 48h application groups, which may indicate that short-term stimulation does not invoke chondrogenic morphogenesis in muscle tissue. This was also reflected in the relative expression levels of *ACAN*, *SOX9* and *Col2α1*. Jelicet al.[181] demonstrated that prolonged application of OP-1 promoted better regeneration of articular cartilage in chondral defects compared to a single intra-articular injection. Therefore, the creation of a chondrogenic morphogenesis environment depends on the sustained role of growth factors. However, *in vivo* experiments performed by Neol et al.[182] revealed that short-term *BMP-2* expression was sufficient to induce the osteochondral differentiation under a Tet-Off system. As such, there may be other factors that affect the stimulation of a single application to the muscle tissue and is worth considering. Ren et al.[183], for instances, also used muscle tissue fragments and modified the cells of the tissue using adenoviral genetic technology, yet postulated that tissue wide change would only occur on the surface of fascia and surface of the tissue. As such it was assumed that there was

a possible flaw in the single 48h morphogen application administrations in which the release kinetics or possible signal transition did not effectively reach a functional concentration to cause a relevant stimulus.

4.4 Chondrogenesis induction: synergistic morphogen combinations

Toh et al.[184] initially demonstrated that BMP subfamily could induce the formation of bone and cartilage in ectopic sites and act as autocrine and/or paracrine factor to regulate the development of bone and cartilage. They also described that BMP-2 regulated the maturation of mesenchymal progenitors and promoted the synthesis of the chondrocyte matrix. By comparing the different effects of BMP-2, -4 and -6, Sekiya et al.[185] suggested using BMP-2 to produce polysaccharide-rich cartilage quantities. Schmal et al.[186] demonstrated the importance of BMP-2 in cartilage repair and maintenance through *in vivo* experiments. The present study results were in agreement with these published opinions in which it was speculated that BMP was the inducer of chondrogenic morphogenesis. On day 14, semi-quantitative histomorphometric analysis of IHC indicated that the group where BMP-2 was applied alone continuously had the highest MOD value, but then dropped sharply. The results of qRT-PCR showed the same pattern. The relative expressions of chondrogenic-related genes, such as *ACAN*, *SOX9* and *Col2α1* on day 7 in the group applied BMP-2 continuously were significantly higher than that in the other groups, but there was also a sharp decline in the subsequent detections. Van Beuningen et al.[187] pointed out that BMP-2 stimulated

chondrocyte proteoglycans were synthesized earlier and stronger than TGF-β isoforms, but the duration was shorter. Additionally, several researchers have demonstrated that a short period of BMP-2 expression or co-incubation time was necessary to induce bone regeneration or promote the osteogenic differentiation of MSCs *in vitro*[188-191], which indicated that BMP-2 might not only serve as the inducer in the chondrogenesis, but also in osteogenesis[192,193].

Hellingman et al.[194] demonstrated that phosphorylation of both SMAD 2/3 and SMAD 1/5/8 was essential for initiating chondrogenic differentiation and these SMADs maintained their activity in differentiated MSCs, while only SMAD 2/3 was found in native articular cartilage. This evidence pointed out that induced articular chondrogenesis could only be maintained through the signaling involving SMAD 2/3 regulated by TGF-β isoforms and may also explain the higher MOD in TGF-β_3+ OP-1 continuous stimulation group on day 30 and the stronger expression of *ACAN* and *SOX9* on day 14 and 30 in groups applied OP-1 + TGF-β_3 and BMP-2 + OP-1 + TGF-β_3 continuously, respectively. While OP-1 up-regulated chondrocyte metabolism[195-197], stimulated only cartilage-specific extracellular proteins[198-200] and generated normal functional proteoglycans[201], it explained why the expression of *Col2α1* was stable in the experimental group containing OP-1. By analyzing the results of gene expressions over time in different experimental groups, the extreme decline from day 7 to 14 in the group using BMP-2 alone continuously over 30 days, was prevented by different combinations with the other growth factors. Therefore, BMP-2 alone may have been

sufficient to evoke chondrogenesis, but TGF-β_3 and OP-1 are critical extra requirements for articular cartilage tissue engineering, especially in the middle and late stages of induction, as TGF-β_3 and OP-1 help maintain the process.

Interestingly, increased MOD and relative expression levels of chondrogenic-related genes induced by BMP-2 alone was inhibited by the addition of TGF-β_3 or/and OP-1, showing an apparent antagonism effect. The possible explanation was that the TGF-β superfamily signal pathways can antagonize each other, although these mechanisms remain unclear[202,203]. First, it was hypothesized that BMP-2 was the most active growth factor during morphogenesis at the initial stage of addition until 7 days. Along this line of thought, it can be deduced that both SMAD 2/3 (regulated by TGF-βs) and SMAD 1/5/8 (regulated by BMPs) need to interact with SMAD 4, which is a cofactor and prerequisite for the regulation of target genes transcription in which the competition led to antagonism between TGF-βs and BMPs[119]. The functions of TGF-βs in cartilage were reviewed by Wang et al.[100], which indicated that TGF-βs could participate in both TGF-βs (SMAD 2/3) and BMPs (SMAD 1/5/8) signals. Kraan et al.[92] elaborated a similar viewpoint that besides binding to ALK 5 *via* canonical pathway (SMAD 2/3), TGF-βs can also bind to ALK1 and ALK2 in some cell types to activate SMAD 1/5/8, thus activating the BMPs pathway. However, the extent to which TGF-βs utilized BMPs pathways in the present culture systems needs further evaluations. Moreover, Gronroos et al.[204] described a relevant mechanism where TGF-βs inhibited BMP signaling by forming an inhibitory complex, SMAD 1/5-SMAD 3, involved in the inhibition of

phosphorylation. Hence, TGF-βs and BMPs may serve sequentially in the regulation of cartilage differentiation[205,206] and the antagonistic, as well as synergistic activities, which were dependent on the differentiation stage[162,194,207]. Because the current results have revealed the antagonism between the three growth factors in the early stages of tissue culture, in order to make up an optimal protocol, more details are needed and considered, such as erasing TGF-βs in the early stage and sequentially adding growth factors as culture time progress or removing them as required to generate a consistent articular cartilage formation response.

4.5 The limitations

In terms of limitations in the study, first, the detection mainly focused on and was limited to, chondrogenesis. However, the expressions of *Col1α1*, whose product also makes up more than 90% of bone matrix[167], was recovering after day 7 in the present study. Although the evidence was insufficient, it was probable that not only did chondrogenesis occur, but also osteogenesis-related reactions were in progress in this culture system, which may attribute to the diverse response raised by various cell types in muscle tissue receiving the same stimulation. Many studies demonstrated that the response to the intervention of TGF-β superfamily proteins depended on the culture system or models used[208-210]. For these reasons, the spatial and temporal patterns of these two types of morphogenesis processes in the present study cannot be fully clarified.

Second, although previous literature recommended certain growth factor concentrations, more experiments based on the gradient concentration are needed. Some investigators have shown that the biphasic effects of TGF-β superfamily can affect DNA synthesis[208,209,211]. Yang et al.[212] also demonstrated that BMP-2 served as a double-edged sword in the osteogenic differentiation induction by TGF-β isoform activated kinase 1 in MSCs, which is regulated by different concentration of BMP-2. Additionally, the expressions of *Col1α1* and *Col10α1* were related to SMAD 7, which acted as an intracellular inhibitor of BMP and TGF-β isoform signaling and expression depends on the type of TGF-β signaling[213-215]. Therefore, it cannot be accurately concluded whether an inappropriate growth factor concentration caused the suppressed results of *Col1α1*, *Col10α1* and even *ALP*.

Compared with fresh tissue, the control group also showed up-regulation of genes, such as *ACAN* and *SOX9*, although significantly lower than the corresponding experimental groups stimulated continuously, indicating the chondrogenesis effect observed in the present study may not be wholly dependent on the application of growth factors. Fahlgren et al.[216] demonstrated that the expression of *BMP-7* in rabbit knee joint cartilage increased after a capsular incision. Several studies also showed that micro-fractures in cartilage possess a chondroprotective effect and stimulate cartilage repair [116,217,218]. Therefore, it has to also be considered whether the damage caused by biopsy punches in the harvest of relevant muscle tissue fragments, in the present experiment,

was a mechanical stimulus that brought about an increase in gene expression due to a local reaction. Future experiments will also thus need to assess the importance of mechanical[219] in conjunction with the biological stimulation in muscle tissue based culture models seeking to generate articular cartilage.

The fourth limitation of this study was that no corresponding antagonist, to BMPs or TGF-β_3, was applied. The chondrogenesis observed was induced by the signals raised from multiple extracellular ligands simultaneously *in vitro* and via the different pathways, then integrating and interpreting them to respond appropriately[204]. However, the extent of specific signaling pathways functioning in the morphogenesis cannot be verified. Tsumaki et al.[220] and Pathi et al.[221] demonstrated the necessity of BMP signaling pathway by using Noggin to achieve specific blockade[222,223]. Moreover, many studies have shown that in addition to SMAD 2/3, TGF-βs can also phosphorylate SMAd1/5/8 which hitherto considered unique to BMPs, so the use of antagonists is required to prove whether TGF-βs had an impact through this pathway in our culture system in future studies.

4.6 Future studies

Given the limitations of the present study and the needs of the development of disciplines, numerous additional experiments have to be considered. First, more osteogenesis-related detections should be included in the experimental design. For

example, the Movat's pentachrome stain, which assesses for collagen associated with chondrogenesis and osteogenesis, elastic fibers, muscle and other connective tissue[224] may address the temporal and spatial patterns of different morphogenesis. In addition, according to the recovery of *Col1α1* at the final detection day 30, the time points should be increased and extended appropriately to generate more information about whether osteogenesis takes over after this time point or is a simple regulatory pattern that occurs once every few days. Additionally, five factors were previously proposed to elucidate bone and cartilage engineering development[225]: 1) cells directly involved in morphogenesis, 2) matrices produced by the cells, 3) body fluid, 4) regulators of cellular activities and morphogenesis progress and 5) biomechanical dynamics. Hence, the impacts of biomechanics on chondrogenic or osteogenic morphogenesis is another important aspect worth studying. To further analyze whether cells with various phenotypes respond differently to growth factors, alone or in combinations, it may also be necessary to consider a single cell type, such as muscle-derived stem cells, muscle fibroblasts or myoblasts. Finally, by increasing the application of antagonists, a more comprehensive understanding of growth factors can be achieved: whether there exists a specific pathway that is central in the articular chondrogenesis process and what is the exact functional time point at which different growth factors need to be added to maintain the relevant process without it going off on a tangent.

5. Conclusions

Muscle tissue was shown to be a viable model in this chondrogenic induction study. The application of the member of TGF-β supergene family, alone or in combinations, can induce chondrogenesis in this tissue model, with the initial prospect being towards of hyaline cartilage composed primarily of Col2α1. Although the experiment attempted to achieve a more economic-efficiency induction scheme by 48h morphogen stimulation withdrawal study, it has shown that single stimulation of growth factor was insufficient to evoke the relevant response, in which only continuous morphogen application could generate the desired response in which it was critical the type of morphogens was used and when they were used. In terms of different effects caused by growth factors, alone or combinations, BMP-2 alone was sufficient to initiate chondrogenesis, which could be inhibited by the addition of TGF-β_3 or/and OP-1. However, the TGF-β_3 and OP-1 were necessary for cartilage tissue engineering, especially in the middle and late stages of chondrogenic induction. Although limitations still exist, the present study shows the importance of TGF-β supergene family proteins in tissue engineering, providing a novel approach and strategy for the production of engineered cartilage in the future.

6. Abbreviations

ACTB	Actin beta
ACAN	Aggrecan
ALK	Activin receptor-like kinases
ALP	Alkaline phosphatase
BMP	Bone morphogenetic protein
CDS	Coding sequences
CNRQ	Calibrated normalized relative quantities
Col10α1	Collagen type X alpha 1
Col1α1	Collagen type I alpha 1
Col2α1	Collagen type II alpha 1
Col4α1	Collagen type IV alpha 1
Cq	Cycle quantification
Ct	Cycle threshold Value
DI water	Deionized water
ECM	Extracellular matrix
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDFs	Growth and differentiation factors
IHC	Immunohistochemistry
IOD	Integrated optical density value
L	Liter

MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
mL	Milliliter
mM	Millilimole
mm	Millimeter
MOD	Mean optical density value
MSCs	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
ng	Nanogram
OP-1	Osteogenic protein 1
POLR2e	RNA polymerase II subunit e
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
RNA28S4	RNA-28S ribosomal 4
RPL13α	Ribosomal protein L13α
RPLP0	Ribosomal protein lateral stalk subunit P0
R-SMADs	Receptor-regulated SMAD proteins
SDHA	Succinate dehydrogenase complex flavoprotein sub-unit A
SOX 9	Sex-determining region Y (SRY)-box 9
TBP	TATA-binding protein
TGF-β	Transforming growth factor beta
VEGF-A	Vascular endothelial growth factor A
WP	Washing puffer

ΔG	Gibbs standard free-energy change
µL	Microliter
µmol	Micromole

7. Reference

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8. Appendixes

Appendix 1. Statistical comparison of CNRQs of different genes at adjacent time points in experimental groups stimulated for 48h

Group	Time (day)	Genes							
		Col1α1	***Col2α1***	***Col4α1***	***Col10α1***	***ACAN***	***ALP***	***SOX9***	***VEGF-A***
Control	7	0.17±0.21	0.22±0.10	0.49±0.12	-0.66±0.12	0.88±0.10	-0.79±0.0	0.13±0.07	0.12±0.1
	14	0.17±0.19	-0.21±0.1	0.27±0.06	-0.21±0.28	0.98±0.12	-1.06±0.0	0.23±0.01	0.88±0.1
	30	0.58±0.17	-0.16±0.0	-0.11±0.0	-0.46±0.28	1.31±0.47	-1.30±0.1	0.70±0.09	1.26±0.1
	7 vs. 14 P-value	0.9987	**0.0001**	**0.0022**	**0.0296**	0.8527	**0.0145**	0.0513	**<0.0001**
	14 vs. 30 P-valu	**0.014**	0.7822	**<0.0001**	0.2635	0.2044	**0.0331**	**<0.0001**	**0.0004**
BMP-2	7	-0.26±0.12	0.55±0.17	-0.26±0.12	-0.36±0.94	0.76±0.16	-0.85±0.12	0.10±0.10	0.11±0.12
	14	0.35±0.11	-0.21±0.19	0.35±0.11	-0.44±0.10	1.08±0.61	-0.96±0.10	0.29±0.12	-0.20±0.1
	30	0.67±0.15	-0.27±0.14	0.67±0.15	-0.64±0.00	1.46±0.49	-1.04±0.36	-0.46±0.28	0.61±0.18
	7 vs. 14 P-value	**<0.0001**	**<0.0001**	**0.0023**	0.9753	0.3628	0.6808	0.0881	**0.0002**
	14 vs. 30 P-valu	**0.0021**	0.9829	**<0.0001**	0.8971	0.2344	0.8876	**<0.0001**	**<0.0001**
TGF-β_3	7	0.20±0.15	0.58±0.26	0.20±0.15	-0.63±0.27	0.43±0.10	-1.09±0.05	-0.02±0.16	0.18±0.06
	14	0.29±0.13	-0.50±0.20	0.29±0.13	-0.34±0.04	0.44±0.23	-1.17±0.14	0.14±0.09	-0.13±0.0
	30	0.72±0.10	-0.21±0.12	0.72±0.10	-0.76±0.09	1.22±0.42	-1.33±0.07	-0.16±0.08	0.65±0.12
	7 vs. 14 P-value	0.6166	**<0.0001**	0.6166	0.0686	0.9994	0.6855	**0.0395**	**<0.0001**
	14 vs. 30 P-valu	**<0.0001**	0.0973	**<0.0001**	**0.0231**	**<0.0001**	0.1673	**<0.0001**	**<0.0001**
OP-1	7	-0.08±0.12	0.53±0.18	-0.08±0.12	-0.85±0.12	1.14±0.39	-1.04±0.17	0.05±0.20	0.14±0.10

	14	0.31±0.09	-0.35±0.16	0.31±0.09	-0.05±0.07	0.59±0.29	-0.98±0.21	0.09±0.03	0.21±0.09
	30	0.64±0.26	-0.22±0.15	0.64±0.26	-0.65±0.12	1.61±0.28	-1.13±0.20	-0.41±0.13	0.74±0.09
	7 vs. 14 P-value	**0.0009**	**<0.0001**	**0.0009**	**<0.0001**	**0.0044**	0.945	0.8438	0.7278
	14 vs. 30 P-valu	**0.0041**	0.3794	**0.0041**	**<0.0001**	**<0.0001**	0.5032	**<0.0001**	**<0.0001**
BMP-2	7	-0.11±0.18	-0.22±0.22	-0.11±0.18	-0.81±0.43	1.23±0.40	-1.11±0.23	0.01±0.04	0.12±0.05
+	14	0.24±0.12	-0.42±0.01	0.24±0.12	-0.36±0.34	0.03±0.10	-1.32±0.02	-0.04±0.08	-0.03±0.1
TGF-β_3	30	0.60±0.18	-0.20±0.16	0.60±0.18	-0.49±0.01	1.23±0.19	-1.15±0.10	-0.48±0.23	0.61±0.08
	7 vs. 14 P-value	**0.0001**	0.3083	**0.0001**	0.1244	**<0.0001**	**0.0378**	0.8091	**0.0013**
	14 vs. 30 P-valu	**0.0001**	0.213	**0.0001**	0.8163	**<0.0001**	0.1355	**<0.0001**	**<0.0001**
BMP-2	7	-0.54±0.28	0.58±0.19	-0.54±0.28	-0.63±0.20	1.34±0.39	-1.23±0.11	-0.03±0.16	0.13±0.21
+	14	0.24±0.09	-0.36±0.34	0.24±0.09	-0.17±0.35	0.45±0.40	-1.22±0.09	0.13±0.10	-0.01±0.0
OP-1	30	0.73±0.17	0.25±0.08	0.73±0.17	-0.01±0.26	1.17±0.19	-0.73±0.20	-0.34±0.08	0.62±0.10
	7 vs. 14 P-value	**<0.0001**	**<0.0001**	**<0.0001**	0.0529	**<0.0001**	0.9986	**0.0247**	0.1389
	14 vs. 30 P-valu	**<0.0001**	**<0.0001**	**<0.0001**	0.6742	**0.0002**	**<0.0001**	**<0.0001**	**<0.0001**
	7	-0.15±0.09	0.33±0.27	-0.15±0.09	NA	0.71±0.52	-1.51±0.15	-0.33±0.06	0.04±0.13
TGF-β_3	14	0.33±0.31	-0.55±0.14	0.33±0.31	-0.49±0.21	0.57±0.46	-1.26±0.12	0.28±0.31	0.06±0.10
+	30	0.83±0.13	0.11±0.28	0.83±0.13	-0.79±0.04	1.09±0.55	-0.80±0.15	-0.26±0.04	0.60±0.11
OP-1	7 vs. 14 P-value	**<0.0001**	**<0.0001**	**<0.0001**	NA	0.9055	**0.0091**	**<0.0001**	0.979
	14 vs. 30 P-valu	**<0.0001**	**<0.0001**	**<0.0001**	0.0526	0.0914	**<0.0001**	**<0.0001**	**<0.0001**
BMP-2	7	-1.14±0.54	0.14±0.24	-1.14±0.54	NA	0.82±0.60	-1.09±0.11	0.17±0.14	0.21±0.07
+	14	0.33±0.10	-0.24±0.32	0.33±0.10	-0.27±0.17	0.91±0.53	-0.73±0.06	0.36±0.17	-0.11±0.0

TGF-β_3	30	0.54±0.38	0.41±0.12	0.54±0.38	0.05±0.50	0.77±0.15	-0.52±0.15	-0.34±0.11	0.26±0.04
+	7 vs. 14 P-value	**<0.0001**	0.0634	**<0.0001**	NA	0.9653	**0.0115**	0.0598	**<0.0001**
OP-1	14 vs. 30 P-valu	0.5243	**0.0007**	0.5243	0.2226	0.8932	0.2308	**<0.0001**	**<0.0001**

All data are presented as mean ± standard deviation. The comparation was performed using ANOVA. We define $p<0.05$ as a statistically significant difference (in bold). CNRQ: Calibrated normalized relative quantity, *Col1α1*: *Collagen Type I Alpha 1*, *Col2α1*: *Collagen Type II Alpha 1*, *Col4α1*: *Collagen Type IV Alpha 1*, *Col10α1*: *Collagen Type X Alpha 1*, *ACAN*: *Aggrecan*, *ALP*: *Alkaline phosphates*, *SOX9*: *SRY (Sex Determining Region Y)-Box 9*, *VEGF-A*: *Vascular endothelial growth factor A*. NA: Not available.

Appendix 2. Statistical comparison of CNRQs of different genes at adjacent time points in experimental groups stimulated continuously

Group	**Time (day)**	**Genes**						
		Col1α1	***Col2α1***	***Col4α1***	***ACAN***	***ALP***	***SOX9***	***VEGF-A***
Control	7	0.17±0.21	0.22±0.10	0.49±0.12	0.88±0.10	-0.79±0.0	0.13±0.07	0.12±0.10
	14	0.17±0.19	-0.21±0.1	0.27±0.06	0.98±0.12	-1.06±0.0	0.23±0.01	0.88±0.12
	30	0.58±0.17	-0.16±0.0	-0.11±0.0	1.31±0.47	-1.30±0.1	0.70±0.09	1.26±0.11
	7 vs. 14 P-value	0.9987	**0.0001**	**0.0022**	0.8527	**0.0145**	0.0513	**<0.0001**
	14 vs. 30 P-valu	**0.014**	0.7822	**<0.0001**	0.2044	**0.0331**	**<0.0001**	**0.0004**
BMP-2	7	-0.66±0.1	4.71±0.12	0.58±0.10	3.91±0.13	1.83±0.06	0.72±0.13	0.08±0.08
	14	-0.34±0.1	0.67±0.08	0.43±0.05	2.15±0.18	-0.86±0.1	0.40±0.07	0.82±0.13
	30	0.23±0.14	0.04±0.75	0.20±0.09	2.04±0.26	-1.12±0.1	0.26±0.05	1.09±0.07
	7 vs. 14 P-value	**0.025**	**<0.0001**	**0.0341**	**<0.0001**	**<0.0001**	**0.0004**	**<0.0001**
	14 vs. 30 P-valu	**0.0004**	0.1382	**0.002**	0.6802	**0.0194**	0.0725	**0.0024**
TGF-β_3	7	-0.37±0.2	0.53±0.47	0.41±0.15	1.98±0.18	-0.84±0.1	0.34±0.15	1.01±0.15
	14	-0.38±0.1	0.87±0.06	0.19±0.06	2.50±0.15	-0.92±0.1	0.39±0.06	1.03±0.08
	30	0.86±0.07	NA	0.11±0.03	2.66±0.20	-1.29±0.1	0.36±0.05	0.92±0.11
	7 vs. 14 P-value	0.9821	0.3104	**0.0104**	**0.0016**	0.7578	0.6964	0.9504
	14 vs. 30 P-valu	**<0.0001**	NA	0.3744	0.3406	**0.0085**	0.8434	0.3559
OP-1	7	-0.62±0.2	0.57±0.09	0.30±0.10	1.57±0.33	-0.78±0.1	0.43±0.04	1.10±0.02
	14	-0.01±0.0	0.73±0.16	0.50±0.06	2.78±0.11	-0.92±0.2	0.36±0.05	0.96±0.20
	30	0.14±0.11	0.95±0.13	0.43±0.09	2.39±0.11	-1.07±0.1	0.32±0.07	1.03±0.09
	7 vs. 14 P-value	**0.0001**	0.2433	**0.0069**	**<0.0001**	0.441	0.1404	0.2312

	14 vs. 30 P-valu	0.3004	0.1286	0.3826	**0.0331**	0.4426	0.4651	0.6737
BMP-2	7	-0.60±0.0	0.68±0.03	0.58±0.05	2.49±0.08	-0.77±0.1	0.52±0.07	0.98±0.02
+	14	-0.43±0.0	1.12±0.18	0.12±0.13	2.17±0.06	-1.04±0.1	0.48±0.05	1.09±0.06
TGF-β_3	30	0.05±0.10	0.09±0.48	0.43±0.15	2.23±0.10	-1.34±0.0	0.31±0.03	1.34±0.02
	7 vs. 14 P-value	**0.0219**	0.1023	**0.0001**	**0.0001**	**0.0126**	0.4726	**0.0009**
	14 vs. 30 P-valu	**<0.0001**	**0.0022**	**0.004**	0.5367	**0.004**	**0.0005**	**<0.0001**
BMP-2	7	-0.75±0.0	0.67±0.14	0.61±0.07	1.75±0.23	-1.05±0.2	0.40±0.01	1.09±0.06
+	14	-0.62±0.2	0.82±0.16	0.25±0.22	2.29±0.17	-0.85±0.1	0.36±0.04	0.90±0.15
OP-1	30	-0.10±0.0	NA	0.02±0.01	1.99±0.17	-1.38±0.0	0.28±0.10	1.09±0.01
	7 vs. 14 P-value	0.2641	0.3566	**0.0027**	**0.0018**	0.1791	0.5903	**0.0171**
	14 vs. 30 P-valu	**<0.0001**	NA	**0.0455**	0.0628	**0.001**	0.1073	**0.016**
	7	-0.67±0.1	0.73±0.09	0.43±0.10	1.53±0.21	-0.77±0.1	0.37±0.09	1.04±0.09
TGF-β_3	14	-0.21±0.1	0.69±0.17	0.43±0.13	2.84±0.24	-0.88±0.0	0.51±0.04	0.93±0.03
+	30	0.47±0.07	0.93±0.20	0.41±0.11	2.37±0.18	-0.81±0.1	0.40±0.03	1.24±0.04
OP-1	7 vs. 14 P-value	**0.0002**	0.931	0.9988	**<0.0001**	0.3732	**0.01**	**0.0238**
	14 vs. 30 P-valu	**<0.0001**	0.1528	0.9636	**0.0109**	0.7073	**0.0305**	**<0.0001**
BMP-2	7	-0.80±0.1	0.75±0.12	0.72±0.11	2.49±0.18	-0.49±0.2	0.63±0.17	1.16±0.02
+	14	-0.25±0.0	0.79±0.49	0.69±0.05	2.25±0.07	-0.64±0.3	0.46±0.13	1.12±0.06
TGF-β_3	30	0.58±0.10	0.84±0.09	0.32±0.05	3.27±0.13	-1.26±0.1	0.49±0.05	1.33±0.01
+	7 vs. 14 P-value	**<0.0001**	0.9814	0.8555	**0.0423**	0.6518	0.1334	0.1281
OP-1	14 vs. 30 P-valu	**<0.0001**	0.9764	**<0.0001**	**<0.0001**	**0.0061**	0.9563	**<0.0001**

All data are presented as mean ± standard deviation. The comparation was performed using ANOVA. We define $p<0.05$ as a statistically significant difference (in bold). CNRQ: Calibrated normalized relative quantity, *Col1α1*: *Collagen Type I Alpha 1*, *Col2α1*: *Collagen Type II Alpha 1*, *Col4α1*: *Collagen Type IV Alpha 1*, *ACAN*: *Aggrecan*, *ALP*: *Alkaline phosphates*, *SOX9*: *SRY (Sex Determining Region Y)-Box 9*, *VEGF-A*: *Vascular endothelial growth factor A*. NA: Not available.