UNIVERSITÀ DI PARMA

UNIVERSITA' DEGLI STUDI DI PARMA

Dottorato di ricerca in Medicina Molecolare

Ciclo XXXIII

The role of stanozolol in the stimulation of cellular mechanisms for osteochondral regeneration

Ruolo dello stanozololo nella stimolazione dei meccanismi cellulari per la rigenerazione osteocondrale

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Anni Accademici 2017/2018 - 2019/2020

"Io descriverò il comportamento della natura ma se a voi questo comportamento non piace, il vostro processo di comprensione ne risulterà intralciato. […] il punto essenziale non è se una teoria piaccia o non piaccia, ma se fornisca previsioni in accordo con gli esperimenti. La ricchezza filosofica, la facilità, la ragionevolezza di una teoria sono tutte cose che non interessano. […] mi auguro quindi che riuscirete ad accettare la natura per quello che è: assurda."

(Richard Phillips Feynman)

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Foreword

Bone and cartilage tissues are both parts of the group of highly specialized connective tissues. Bone and cartilage cells stem from differentiation phenomena that occur in mesenchymal stem cells. Since bone regeneration is a long and complex process and cartilage basically lacks regenerative capability, the research which aims to establish treatments that can improve or induce these tissues' regenerative mechanisms is of paramount importance.

Stanozolol is one of the many molecules that are of interest in this context. Stanozolol is a steroid whose capability to augment regeneration both in bone and cartilage has already been established but whose pathway of action is still not univocally described or known with certainty.

The current manuscript is the description of five experiments aimed at showing how stanozolol improves osteochondral differentiation, especially when accompanied by a co-treatment able to trigger the BMP2 pathway. Afterwards, we investigated the receptors involved in stanozolol signalling and made a hypothesis about which pathway was involved.

In the first chapter, we address the difficulties related to chondrocyte cell culture in a two-dimensional culture and the peculiarities of chondrocytes in a three-dimensional culture; therefore, the appendix to the first chapter highlights how stanozolol can improve cartilage matrix related genes' expression in a micromass cell culture. The subject of the second chapter is osteogenesis, and this chapter shows not only how stanozolol improves osteogenesis markers' gene expression, but also how the efficacy of the treatment can be augmented if accompanied by CD44 stimulation. The third chapter is dedicated to the identification of steroid receptors with which stanozolol interacts, and it strives to describe how stanozolol functions. The appendix to the third chapter includes a further investigation of hypotheses made about stanozolol's pathway of action and proposes a final hypothesis about how stanozolol induces osteochondral differentiation, relying both on the literature and on the outcomes of the described experiments.

Prefazione

I tessuti osseo e cartilagineo fanno parte dei tessuti connettivi altamente specializzati; le cellule che li costituiscono originano dai processi differenziativi a cui vanno incontro le cellule staminali mesenchimali. Dal momento che la rigenerazione ossea è un processo lungo e complesso e che la cartilagine manca sostanzialmente della capacità di rigenerare, la ricerca volta a stabilire trattamenti che possano migliorare o indurre fenomeni di natura rigenerativa, è di fondamentale importanza.

Tra le molte molecole che possono essere di interesse nel detto contesto c'è lo stanozololo, uno steroide la cui capacità di migliorare la rigenerazione ossea e cartilaginea è già stata stabilita ma il cui pathway d'azione non è stato ancora univocamente descritto o conosciuto con certezza.

Questo testo è la descrizione di cinque esperimenti che hanno la volontà di mostrare, a cominciare dalle peculiarità della coltura bidimensionale di condrociti, come lo stanozololo possa migliorare il differenziamento osteocondrale specialmente quando accompagnato da un co-trattamento che sia in grado di stimolare l'azione di BMP2. Alla fine il lavoro raggiunge il suo scopo nel tentativo di chiarire quali siano i recettori con cui interagisce lo stanozololo e cercando di ipotizzare attraverso quale pathway questo svolga la sua funzione.

Nel primo capitolo sono affrontate le difficoltà correlate alla coltura bidimensionale di condrociti e le particolarità legate alla loro coltura tridimensionale; dopo questo, l'appendice al primo capitolo evidenzia come lo stanozololo, in una coltura di micromasse, possa aumentare l'espressione di geni codificanti per elementi della matrice cartilaginea. Nel secondo capitolo l'argomento diventa l'osteogenesi ed è mostrato non solo come lo stanozololo migliori l'espressione di marker osteoidi, ma anche come l'efficacia del trattamento venga aumentata dalla stimolazione di CD44. Nel terzo capitolo è rivelato quale recettore steroideo sia coinvolto nell'interazione con lo stanozololo ed è presente un primo tentativo di descrivere la funzione del detto steroide; nell'appendice al terzo capitolo l'ipotesi fatta circa il meccanismo d'azione dello stanozololo è ulteriormente investigata e, infine, è esposta l'ipotesi definitiva riguardo il modo in cui questo steroide induce il differenziamento osteocondrale, basandosi sia sulla letteratura che sui risultati sperimentali.

Chapter I

The effects of two- and three-dimensional culture methods in chondrocyte differentiation

Abstract

Aim: This study has three main objectives: the first one is to define a culture medium able to maintain a primary chondrocytes' native phenotype *in vitro* in two-dimensional culture, without letting them dedifferentiate or unintentionally pushing them towards the phenomenon of endochondral ossification. The second purpose is to make a gene expression comparison between the condition previously established and a chondrocyte micromass culture obtained with centrifugation. The final aim is to compare gene expression in micromass cultures obtained with or without centrifugation; this last stage has the objective of identifying the best setting with which to establish micromass cell cultures to test the chondrocyte synthesis of hyaline cartilage. **Materials and methods:** Our cell model consisted of equine primary chondrocytes. In a two-dimensional culture, after 7 days of culture in various media, either in normoxia or hypoxia, gene expressions of COL10A1, SOX9, and COL2A1 were evaluated with real-time PCR. As soon as the micromass cultures were established, differences in terms of gene expressions were evaluated by real-time PCR; the genes analysed were: BMP2, SOX9, COMP, COL2A1, CD44, COL1A1, TGFβ1, HIF1α, and COL10A1. Next, chondrocyte micromass cultures were established, with and without centrifugation, in order to assess whether the mechanical stimulus given by the acceleration could lead to the production of fibrocartilage instead of hyaline cartilage. Gene expression was analysed after 24h hours of chondrogenesis permissive medium treatment, with real-time PCR; the analysed genes were: SOX9, COMP, COL2A1, CD44, COL1A1, TGFβ1, and COL10A1. Statistical evaluation was done via one-way ANOVA, followed by Dunnett's multiple comparisons test in the first stage and via t test in the other two; results were considered significant for p-value < 0.05. **Results:** Medium D (containing DMEM high glucose, 2 mM glutamine, 1% ITS, 0.5 mg/ml albumin, 15 U/ml heparin, 10 ng/ml FGF2, and 1X penicillin/streptomycin) was the medium that showed less induction of COL10A1, maintaining a SOX9 and COL2A1 gene expression equal to the control condition. Upon micromass formation, all the genes analysed were upregulated as compared to the chondrocytes kept in medium D in two-dimensional cell culture. Moreover, no significant differences between gene expressions were found due to the process used for micromass aggregation, except for the significant SOX9 and COMP overexpression in the micromass culture obtained with centrifugation, along with a TGFβ1 underexpression. **Discussion:** Medium D, among the media tested, was the best medium to

culture chondrocytes in two-dimensional culture, both in normoxia or hypoxia. Micromass cell culture triggers the cellular differentiation process, and there is a substantial equivalence between the two methods tested to obtain micromass aggregates.

Introduction

Cartilage is a highly specialized connective tissue; it is nerveless and not vascularized, and it is incapable of regeneration. The liquid component of this tissue constitutes a percentage that ranges between 65 and 80%; its extracellular matrix (ECM) is the part that remains, along with the cellular component, which constitutes about 2%. Hyaline cartilage (such as the articular cartilage) along with fibrocartilage and elastic cartilage is one of the three cartilage types that are present within the body. Articular cartilage's main function is to avoid attrition during articulation movement, and, additionally, this tissue has to guarantee the capacity to endure load stress [1][2].

The cartilage ECM is produced by chondroblasts that, during its deposition, remain enveloped in the tissue itself, differentiating in chondrocytes. The main protein components of cartilage ECM are three types of collagen: type II (COL2), IX (COL9), and XI (COL11). Chondrocytes remain the last cell type within the adult tissue (chondroblasts are found only in the perichondrium), and, while chondroblasts have the goal of producing ECM, the chondrocytes' main function is to uphold the matrix. The matrix maintenance is achieved mainly by the production of proteoglycans and other matrix components that are not collagen related. The chondrocyte ability to guarantee the collagen turnover is very limited (yet COL2 has a half-life of about 117 years) [1][2][3].

Considering the organism's failure to overcome the cartilage damage and the fact that this damage can lead progressively to osteoarthritic phenomena [4], the definition of methods that may be a valid option for solving the problem is of paramount importance. In contrast to methods such as autologous transplantation or bone marrow stimulation [5], a potential and promising alternative might well be the substitution of the damaged tissue with functional tissue obtained from autologous primary chondrocytes or bone marrow mesenchymal stem cells (MSCs) [6].

In this specific context, the interest in chondrocyte culture originates from the characteristic phenotype stability of these cells. Nevertheless, it is important to underline the peculiarities related to this specific cell type culture, in which chondrocytes growth and propagation are required.

The most important problems related to the use of chondrocytes are mainly due to the two-dimensional nature of the culture in plates; it is common knowledge how, in two-dimensional culture, chondrocytes tend to dedifferentiate, thus losing their specific native markers, reducing glycosaminoglycan production, and changing their morphology and phenotype into one so-called fibroblastoid cell [3][7][8][9]. Another problem of this cell model lies in the fact that, in the presence of oxygen, chondrocytes tend to become hypertrophic and terminally differentiate going toward endochondral ossification - a phenomenon that, along with intramembranous ossification, is responsible for osteogenesis. Considering all these problems, it is important to maintain chondrocyte culture in a medium that prevents dedifferentiation, and, at the same time, to stop their tendency to become hypertrophic, an event which does not occur under normal conditions in quiescent chondrocytes typical of the articular cartilage [10].

Although it is important to underline how dedifferentiated chondrocytes can be brought back to their former phenotype [7], as well as the fact that high-density cultures can allow for the preservation of the original phenotype even during *in vitro* permanence [3], it is nevertheless important to define the composition of a culture medium that can promote the persistence of the native phenotype in two-dimensional culture. The state of the art medium used in order to avoid the previously discussed drawback is one characterized by the presence of 2% FBS and 1% ITS (insulin-transferrin-selenium mix): this medium appears to allow cellular proliferation and guarantee the permanence of the differentiated phenotype [8][11].

A particularly useful option in terms of studying chondrocyte differentiation is the use of the micromass culture. This method is specifically used in differentiation assays and has a biological meaning that differs when the cells used for this type of culture are chondrocytes rather than others, such as MSCs. In the former case, this culture is used to simulate the adult cartilage environment, while, in the latter case, the aim is to simulate the mesenchymal condensation, a process that, during embryonic development, leads to the anlagen formation [9][12].

Micromasses are a tri-dimensional way to culture cells that provides cell-cell adhesion rather than cell-plastic adhesion. The stimulation achieved by the micromass culture is mediated by the CD44 receptor, the hyaluronic acid (HA) receptor. It usually mediates cell-matrix interaction, but it can also mediate cell-cell interaction [13]. CD44 promotes differentiation through the activation of various factors, such as BMP2 [14],

a ligand of the TGFβ factor family involved both in cartilage and bone differentiation [15][16][17][18]. Micromass cultures can be produced utilizing a number of cells that can range from 250,000 to 500,000, regardless of the type of cells, be they chondrocytes or MSCs [6][19][20].

The problem to address when it comes to the use of micromass culture made out of chondrocytes is the possibility that the centrifugation, necessary to form the required cell pellet, can promote the deposition of fibrocartilage matrix instead of the hyaline cartilage. The literature shows that mechanical stress can lead to the deposition of the former kind of cartilage [21]. To assess which one of the two types of cartilage is produced by chondrocytes, the differential expression of common cartilage matrix markers can be evaluated (For example, fibrocartilage contains more COL1 and less COL2 when compared to hyaline cartilage) [22][23].

Given the aforementioned objectives, this study is divided into three different stages:

- In the first stage, we tested the ability of different media used to culture chondrocytes in a two-dimensional setting in order to sustain a proper duplication rate, while preserving the native phenotype and not allowing the chondrocytes to initiate differentiation or dedifferentiation processes;

- In the second stage, we compared the gene expression of chondrocytes in micromasses obtained with centrifugation to that of cells maintained in a two-dimensional culture with the best medium chosen in the previous stage;

- The third stage was focused on assessing the difference between the gene expression of chondrocytes of micromasses, with and without centrifugation. This test was to verify which one of the two methods is more efficient for producing micromass cultures for experiments aimed at assessing chondrogenesis induction. The control condition was the gene expression of chondrocytes maintained in the best medium, selected during the first stage.

A culture media comparison of chondrocytes in two-dimensional culture

Materials and methods

Equine chondrocyte primary cells

Equine chondrocytes were aseptically harvested from healthy metacarpo/metatarso-phalangeal joints of horses that were obtained from a local slaughterhouse. Not longer than 5 hours after the animal's death, cartilage fragments were dissected with a scalpel from the cartilage surface, and then disinfected with an acqueos solution of 10% povidone-iodine, which was then washed away with PBS. After this step, the cartilage pieces were kept in DMEM high glucose medium with 0.2% collagenase and 2 µg/ml ciprofloxacin. This step, intended to digest the cartilage matrix and free the cells, was carried out at 37° C with an incubation time of one hour each. Every hour the cells freed in the medium were harvested through centrifugation at 300g for 3 minutes, placed in a Petri dish, and then kept in a DMEM/F12 medium supplemented with 30% FBS, 2 mM glutamine, 1% ITS, and 2 µg/ml ciprofloxacin. The collagenase solution was further used to digest the residual cartilage fragments. Next, chondrocytes were cultured in a 75 cm^2 flask with a DMEM high glucose medium supplemented with 10% FBS, 2 mM glutamine, and 1X penicillin/streptomycin.

Bi-dimensional cell culture

20,000 cells/cm^2 were plated in a 6-wells plate and, after 24 h, we replaced the medium with the media to be tested. The media tested, indicated with a capital letter and with an apostrophe in cases where the hypoxia condition was also performed, were prepared as follows:

medium A: DMEM high glucose, 2 mM glutamine, 10% FBS, 1X penicillin/streptomycin;

medium B: DMEM high glucose, 2 mM glutamine, 10% FBS, 15 U/ml heparin, 10 ng/ml FGF2, 1X penicillin/streptomycin;

medium C: DMEM high glucose, 2 mM glutamine, 1% ITS, 0.5 mg/ml albumin, 15 U/ml heparin, 1X penicillin/streptomycin;

medium D: DMEM high glucose, 2 mM glutamine, 1% ITS, 0.5 mg/ml albumin, 15 U/ml heparin, 10 ng/ml FGF2, 1X penicillin/streptomycin;

medium E: DMEM high glucose, 2 mM glutamine, 1% ITS, 10% KO serum, 15 U/ml heparin, 1X penicillin/streptomycin;

medium F: DMEM high glucose, 2 mM glutamine, 1% ITS, 10% KO serum, 15 U/ml heparin, 10 ng/ml FGF2, 1X penicillin/streptomycin.

Cell cultures were kept in a cell incubator at 37° C and 5% CO_2 for 7 days, and media were changed every 2 or 3 days. Hypoxia, when present, was achieved by inserting the 6 wells plates into a plastic bag that, before being closed, was inflated with a gas mixture of 94% N_2, 5% CO_2, and 1% O_2.

Gene expression analysis

Cells were lysed directly in the culture dishes with Trizol (Invitrogen Life-Technologies). Afterwards, the cell slurries were collected into Eppendorf tubes, and the manufacturer's protocol was followed; that is to say, 0.2 ml of chloroform was added to each 1 ml of Trizol solution. Then, samples were vortexed for 15 seconds and incubated at room temperature for 3 minutes. Next, the samples were spun at 12,000g for 15 minutes at 4° C. The upper aqueous phases containing the RNA were mixed with an equal volume of a solution made by 75% of absolute ethanol and 25% of RA1 Buffer from the NucleoSpin RNA XS kit, and, lastly, we adhered to the producer's protocol of NucleoSpin RNA XS kit. The purified RNAs were suspended in 50 µl of RNase free H_2O.

After Nanodrop quantification, 1 µg of RNA for each condition was retro-transcribed with the RevertAid RT kit (Thermo Scientific). To summarize, 0.5 µl of a mixture of random hexamer oligonucleotide primers was added to 4.5 µl of RNA solution. This mix was heated at 70° C for 5 minutes, and then chilled at 4° C for 5 minutes; then, a 15 µl mix made of 4 µl of reaction buffer, 1 µl of RT containing solution, 1 µl of RNase

inhibitor containing solution, 2 µl of dNTPs, and 6 µl of RNase free H_2O was added to the original 5 µl solution. This final mix was heated at 42° C for 60 minutes and then at 70° C for 15 minutes. The 20 µl solution, containing retro-transcripts, was brought to a volume of 200 µl with the addition of 180 µl of nuclease free H_2O.

Gene expression analysis was performed with real-time PCR. In order to perform this assay, a protocol consisting of an initial step at 94° C for 2 minutes was adhered to, followed by 40 cycles with a first denaturation step at 94° C for 15 seconds, followed by 30 seconds primer annealing step, with a temperature dependent on the primers' nucleotide composition, and completed by an extension and reading step at 72° C held for 20 seconds. A final denaturation step was taken to obtain the melt curve of the amplicons, with a temperature rise from 50 to 99° C during a 12 minutes timespan. The mix that was used in this experiment was prepared by combining 7 µl of Luna Universal qPCR Mix (from the New England BioLabs Luna Universal qPCR Master Mix), 3 µl of cDNA solution, 3.44 µl of RNase free water, and 0.56 µl of primer mix (allowing us to have a final concentration of 0.2 µM for each primer).

Primers used in this setting are listed in the table below:

Gene name	Forward primer	Reverse primer
RPL15	CTCTTTCCTTTCCGTCTG	GAGAAGAAATCGCATCAC
COL10A1	TTGTGCTGCTACTGACCTTGA	CACCTTGTTCTCCTCTCACTGAT
SOX9	CTCTGGAGACTGCTGAAC	GTTCTTCACCGACTTCCT
COL2A1	CTGGTGATGATGGTGAAG	GTAACCTCTGTGACCTTTG

Table 1. *Table indicating the primers used to perform real-time PCR assay*

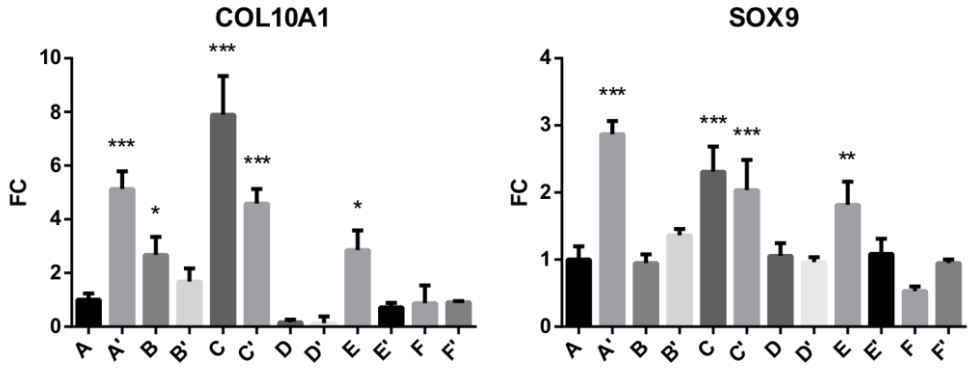
Statistical analysis

The PCR relative quantification was performed using the Pfaffl formula to assess the gene expression variation, and RPL15 was used as the housekeeping gene; this formula, as opposed to the classical $2^{-\Delta\Delta Ct}$ method, takes into account the primers' amplification efficiency, making the data more solid [26]. In the first stage, p-value was assessed via one-way ANOVA, followed by Dunnett's multiple comparisons test, while, in the remaining stages, p-value was calculated with t test; statistical significance was always defined at p-value < 0.05 (with GraphPad Prism 6 software).

Results

Gene expression analysis

Keeping in mind that the media are indicated with a capital letter and that the apostrophe indicates the hypoxia condition, the whole gene expression variations, and their significances, refer to medium A as the control medium in normoxia.



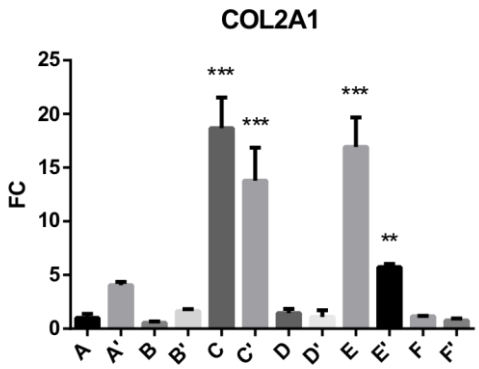


Figure 1. *These graphs represent the fold change of gene expression variation (FC) of COL10A1, SOX9, and COL2A1 genes in the various conditions tested; gene expression was assessed with real-time PCR and analysed with Pfaffl formula [26]; p-value was considered significant for p-value < 0.05 (* p-value ≤ 0.05,** p-value ≤ 0.01, *** p-value ≤ 0.001). It was calculated with one-way ANOVA, followed by Dunnett's multiple comparisons test, performed with GraphPad 6.0. Capital letters indicate the medium used and the presence of an apostrophe indicates that the specific medium condition was applied in hypoxia. All the expression variations were compared with the medium A control in normoxia.*

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The three genes analysed are involved in different stages of the so-called endochondral ossification process. COL10A1, an endochondral ossification marker, is overexpressed in four conditions, in medium C, and in normoxia (fold change = 7.893062, SD = 1.443576, p-value < 0.0001) and hypoxia (fold change = 4.591128, SD = 0.534235, p-value < 0.0001), in medium E in normoxia (fold change = 2.850715, SD = 0.736076, p-value = 0.0107) and, probably due to FBS composition, even in medium A in hypoxia (fold change = 5.128340, SD = 0.659483, p-value < 0.0001). It is important to underline that this gene expression was lower in medium D in normoxia (fold change = 0.1595999, SD = 0.103344) and in hypoxia (fold change = 0.1260919, SD = 0.252279), even though not significantly, due to their SDs. In medium D condition, SOX9 and COL2A1 have the same expression level as in the A control condition (for medium D: SOX9 fold change = 1.056569, SD = 0.188181; COL2A1 fold change = 1.438821, SD = 0.398425; for medium D': SOX9 fold change = 0.9607429, SD = 0.072727; COL2A1 fold change = 1.102123, SD = 0.625707).

Discussion

The genes whose expressions were evaluated are, in general, genes involved in chondrogenesis:

SOX9: the chondrogenesis master switch - it induces the expression of different ECM components. This factor also induces the other two components of the so called SOX trio (SOX5 and SOX6);

COL2A1: the type II collagen α1 chain - it is the cartilage ECM main component;

COL10A1: the type X collagen α1 chain - it is the endochondral ossification marker; even though it appears it is expressed even in the articular cartilage superficial layer [27], it is considered the typical marker of the hypertrophic chondrocyte;

RPL15 was used as the housekeeping gene; this gene codifies for the ribosomal protein L15.

Chondrocyte culture is difficult because of its two-dimensional nature. Chondrocytes, when seeded in a two-dimensional culture, tend to dedifferentiate, while the oxygen presence, as *in vivo*, tends to promote the endochondral ossification pathway. This pathway begins with chondrocyte hypertrophy and is responsible for the axial skeleton development during embryogenesis, for example. During the beginning stages of endochondral ossification, SOX9, through COL2A1, supports hypertrophy, and this phenomenon leads to the activation of marker genes, such as COL10A1.

Taking into account the gene expression fold changes, it appears that, among the media tested, medium D is the best medium to use when it comes to culture chondrocytes *in vitro*, both in hypoxia and normoxia. In medium D, there is no SOX9 or COL2A1 overexpression when compared to the medium A. Furthermore, there is no such thing as SOX9 "dubious" underexpression, as there is in medium F in normoxia. In fact, SOX9 expression is mutually exclusive with RUNX2 gene expression at the beginning of the endochondral ossification process. Medium D COL10A1 expression is substantially absent, especially in the normoxia condition.

The specific function for each one of the medium D components can thus be assessed:

ITS (insulin, transferrin, and selenite mix) - This mix fulfils different tasks: it simulates collagen synthesis, inhibits chondrocytes hypertrophy, functions as an antioxidant, and promotes cell proliferation, etc. When this component is added with 0.5 mg/ml albumin, as in this case, it can be used as a 2% FBS substitute. FBS, in fact, should be avoided because, even if its presence at 2% is considered an important part of the chondrocyte medium, it appears that it can have a dedifferentiating effect in culture [8], other than bringing about contradictory outcomes, probably due to the side-effects caused by its heterogeneous composition in terms of growth factors [25]. FBS is putatively responsible, for the same reasons, for the cell death and lack of aggregation when used in a micromass cell culture [4].

FGF2: This factor increases cell proliferation and proteoglycan synthesis, in addition to reducing fibroblast protein expression in two-dimensional chondrocyte culture [5].

Heparin: When heparin based gels are used for cell culture of chondrocytes that have gone through several steps, it allows for cells' redifferentiation [28]. Furthermore, heparin promotes FGF2 cell binding.

DMEM high glucose: Glucose is a source of cell energy and the precursor molecule of several ECM elements. It also promotes MSCs' survival and inhibits apoptosis, particularly in micromass cell cultures; through the crabtree effect, glucose in high concentration is also responsible for the switch of cell metabolism to one devoted to less oxygen consumption, thus reducing cell respiration. In chondrocyte culture, the higher osmolarity due to 25 mM glucose allows for the maintenance of a chondrocytic phenotype and promotes proteoglycan synthesis [5].

A gene expression comparison between two- and three-dimensional chondrocyte culture

Materials and methods

Cell culture

Primary equine chondrocytes were also used for this set of experiments. In addition to the two-dimensional condition kept in medium D as previously described, micromass culture was carried out with the formation of a single micromass made of 500,000 chondrocytes, placed in a 5 ml conical tube. The micromass was formed from a cell pellet obtained with centrifugation at 500 g for 5 minutes. As soon as the cell pellet closed up on itself, after 48h in culture, the culture was interrupted. Cultures stayed in a cell incubator at 37° C and 5% CO2 for the duration of the experiment, but it is important to underline that, since the micromass tube was hermetically closed, this culture was airtight, and there was no chance for the exchange of air from the inside of the tube to its exterior and vice versa. The composition of the medium used for the micromass formation (which will, from now on, be referred to as the "minimal medium") was DMEM high glucose supplemented with 2 mM glutamine, 1% ITS+ (solution of ITS with an additional dose of albumin that reaches 0.5 mg/ml concentration when added to a medium at 1% of its volume), and 1X penicillin/streptomycin.

Gene expression analysis

RNA isolation and purification was performed as previously described with the exception due to the micromass cultures. In this case, the cell aggregates were first placed in 1 ml of Trizol and then homogenized with CAT X120 homogenizer to obtain a solution to which 0.2 ml of chloroform was added.

Primers used in this setting are listed in the table below:

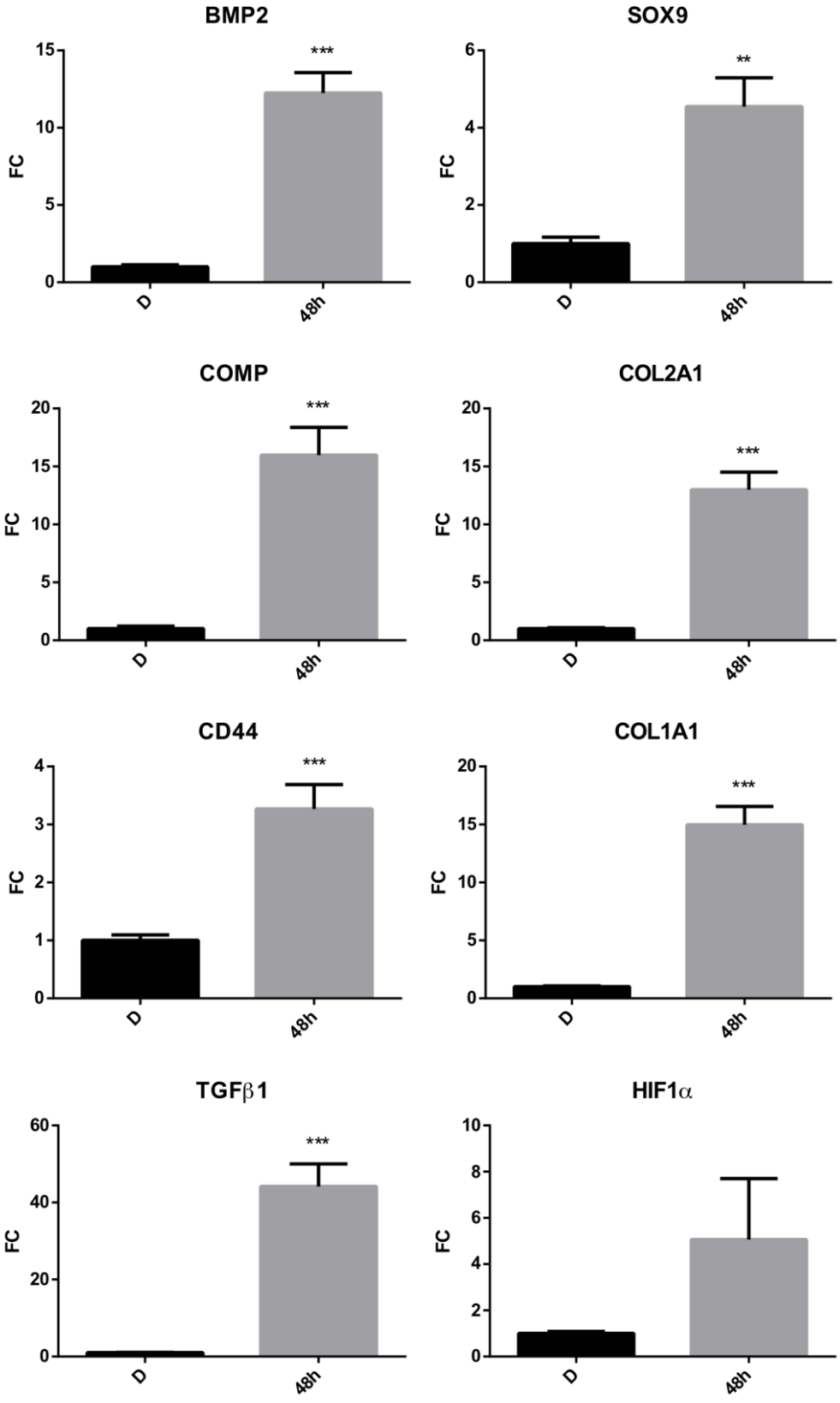
Gene name	Forward primer	Reverse primer
RPL15	CTCTTTCCTTTCCGTCTG	GAGAAGAAATCGCATCAC
COL10A1	TTGTGCTGCTACTGACCTTGA	CACCTTGTTCTCCTCTCACTGAT
SOX9	CTCTGGAGACTGCTGAAC	GTTCTTCACCGACTTCCT
COL2A1	CTGGTGATGATGGTGAAG	GTAACCTCTGTGACCTTTG
BMP2	TTCCACCACGAAGAATCT	ACCTGAAGTTCTGCTGAG
COMP	GACCAGAAGGACACAGAT	TATACCATCGCCATCACT
CD44	AAGACTCCCACTACCAAA	TCCATTGCCATTGTTGAT
COL1A1	GCAAGAACGGAGATGATG	AACCACTGAAACCTCTGT
TGFβ1	CGGACTACTACGCCAAGGA	ATGCTGCTCCACGCTTAAC
HIF1α	CCACTCAGGACACGGACTT	GACGGTAGGAAGAGCAGGTT

Table 2. *Table indicating the primers used to perform real-time PCR assay*

Results

Gene expression analysis

The control condition for these experiments was the chondrocytes kept in a two-dimensional culture in normoxic medium D; the examined condition was the new-formed micromass culture. RNA extraction was performed 48h after cell pellet formation.


BMP2
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48h
COL1A1
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FC
20
15
10
5
0
D
48h
TGFβ1
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FC
60
40
20
0
D
48h
HIF1α
FC
10
8
6
4
2
0
D
48h

Figure 2. *These graphs show the fold of gene expression changes (FC) of BMP2, SOX9, COMP, COL2A1, CD44, COL1A1, TGFβ1, and HIF1α detected in two dimensional culture in respect to micromass induction. Gene expression was assessed with real-time PCR and analysed with Pfaffl formula [26]; p-value was considered significant for p-value < 0.05 (* p-value ≤ 0.05,** p-value ≤ 0.01, *** p-value ≤ 0.001). It was calculated via t test performed with GraphPad 6.0. Medium D was the control condition, while -48h- indicates the micromass gene expression after 48h from the cell pellet formation.*

As we can see, the genes that were analysed, all of which were involved in chondrocyte differentiation, were all significantly overexpressed in the micromass cell culture; the only exception was the higher expression of HIF1α gene (fold change = 5.064414, SD = 2.637294, p-value = 0.0559), that would have been significant with a 90% confidence interval. The COL10A1 graph is not present because this gene was not detected in the micromass culture.

Discussion

Aside from the genes already mentioned, the ones analysed in the second step were:

COMP: The acronym for Cartilage Oligomeric Matrix Protein; it is a cartilage ECM component and typical marker of micromass cell culture;

CD44: A receptor that mediates cell-cell and cell-matrix interactions, and, by means of this function, promotes chondrogenesis;

TGFβ1: A growth factor capable of inducing cell proliferation, cell differentiation, and, in this specific case, chondrogenesis [5]. Although it is mainly involved in repair processes rather than regeneration processes, it is still capable of inducing chondrogenic differentiation, even if not as much as TGFβ2 or TGFβ3 [27];

HIF1α: hypoxia inducible factor - It regulates cell response to low oxygen concentration. It is also an important chondroptosis marker - the chondrocyte alternative apoptotic phenomenon, which is characterized by a contemporary underexpression of COL10A1 and of HIF1α itself;

BMP2: This factor, in this case putatively induced by CD44 stimulation, is a osteogenic and chondrogenic differentiation promoter.

Considering the specific problems related to chondrocyte two-dimensional culture, the need for an alternative type of culture aimed to better initiate differentiation and to study the chondrogenesis process is evident. Micromass cell culture is a specific method for chondrocyte and MSC cultures aimed towards stimulating cellular differentiation, thereby inducing BMP2 gene expression through CD44 activation. BMP2 is capable of promoting both osteogenesis and chondrogenesis. Micromass cultures simulates the environment in which chondrocytes usually dwell in cartilage *in vivo*.

From the graphs and the absence of COL10A1 expression, it appears evident that micromass cell culture is capable of effectively promoting chondrogenesis, making micromass culture the best choice when it comes to a cultural method capable of inducing chondrogenesis. The only gene that did not show a significant overexpression, although it is more than 5 fold higher, is HIF1α; the expression of this gene, along with the absence of the COL10A1 expression, guarantees that no process of chondroptosis is taking place.

A comparison of methods for micromass culture formation

Materials and Methods

Material and methods were previously described

Cell culture

Our aim was to compare two different methods for micromass formation. Micromass cultures were produced either with the same protocol used in the previously described experiments, and, as an alternative, the micromasses were produced without centrifugation, following the protocol described by *Greco et al.* [24]. All told, 500,000 chondrocytes were suspended in 20 µl of DMEM high glucose and put at the bottom of a 5 ml conical tube, and then, after resting for 3h, medium was added slowly, and the pellet present was left alone for 48h, allowing for the micromass formation process. Both micromass cultures, no matter how they

were obtained, were cultured for 48h in the aforementioned minimal medium. After this incubation, a differentiation permissive medium replaced the former one, and cultures were kept in this new condition for 24h until the end of the experiment. The differentiation permissive medium was composed as follows: DMEM high glucose supplemented with 2 mM glutamine, 1% ITS+, 40µg/ml proline, 200 µM ascorbate, 100 µg/ml sodium pyruvate, 10 ng/ml TGFβ1, and 1X penicillin/streptomycin. Cultures were incubated at 37° C and 5% CO2 for the whole duration of the experiment, and, as already stated, the O_2 percentage in the incubator did not make a difference in the micromass cultures, since the conical tubes used for this purpose were airtight. 0.5 mg/ml albumin is about the same concentration of albumin in a medium with 2% FBS; the direct use of albumin is preferable to the use of FBS, as previously stated [4][25].

Results

Gene expression analysis

In these experiments, the gene expression of micromasses made with or without centrifugation refers to the one expressed by chondrocyte bi-dimensional culture with medium D and calculated as fold changes. Once again, COL10A1 was not detected in micromass cultures.

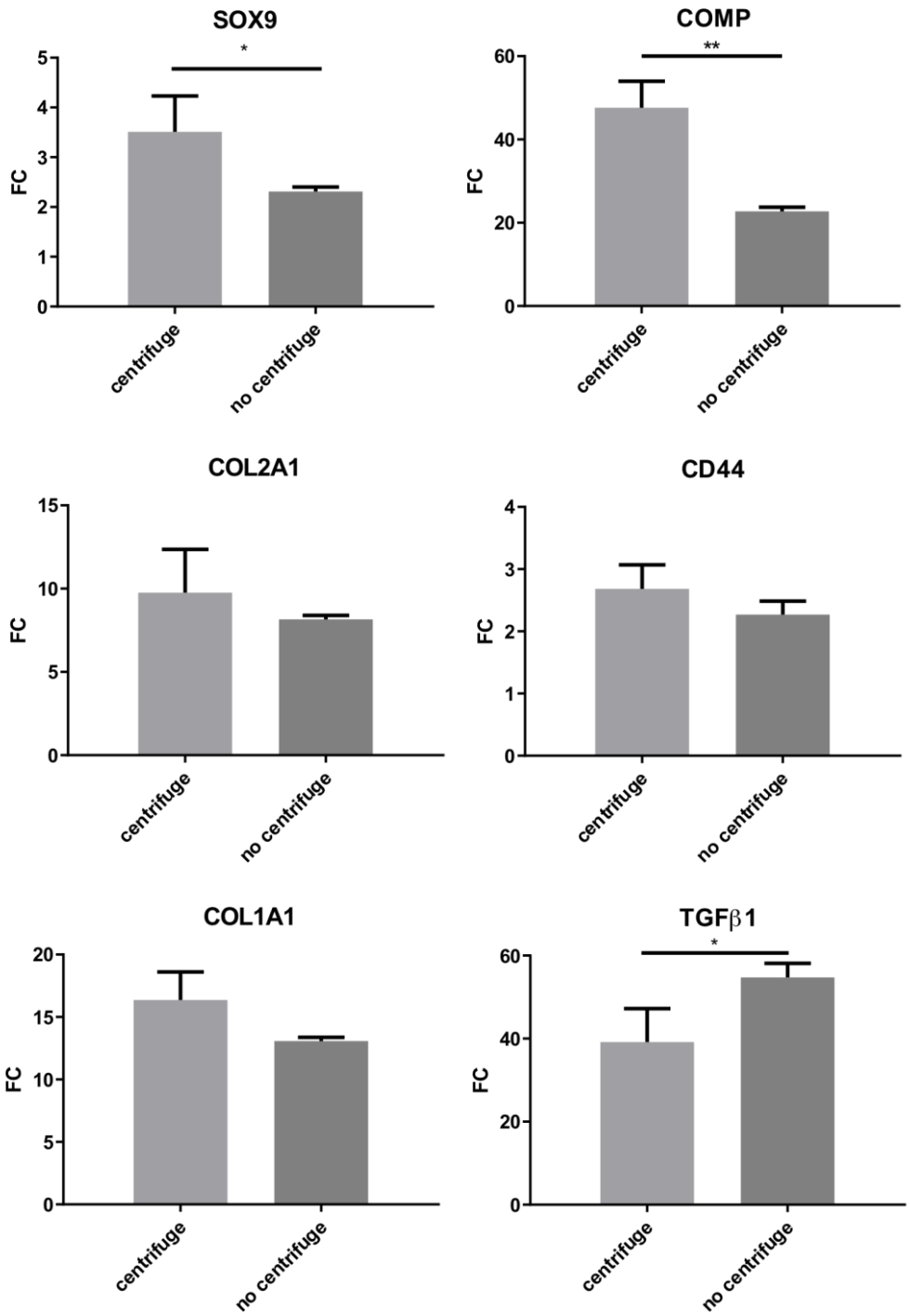


Figure 3. *These graphs show the fold change expression variations (FC) of BMP2, SOX9, COMP, COL2A1, CD44, COL1A1, and TGFβ1 genes for the conditions used; gene expression was assessed with real-time PCR and analysed with Pfaffl formula [26]; p-value was considered significant for p-value < 0.05 (* p-value ≤ 0.05,** p-value ≤ 0.01, *** p-value ≤ 0.001). It was calculated via t test performed with GraphPad 6.0. Gene expression variations are expressed in medium D fold change. The comparison between gene expression fold changes was made between micromass cultures made with or without centrifugation; the medium used was the differentiating one described in the materials and methods section, applied as a condition for 24h after 48h from cell pellets formation.*

The graphs show that significant differences between the two conditions are related to SOX9 (p-value = 0.0460), COMP (p-value = 0.0025), and TGFβ1 (p-value = 0.0368) gene expressions. In the micromass obtained with centrifugation, SOX9 and COMP resulted overexpressed, while TGFβ1 is underexpressed.

Discussion

These experiments made it possibile to evaluate which method is better for producing micromass cultures. The fundamental issue with the centrifugation is that it entails the application of pressure onto cells. As a matter of fact, pressure can promote the deposition of fibrocartilage rather than hyaline cartilage, but, in *in vitro* experiments, the main interest lays in the ability to produce the latter cartilage type. In order to assess which method is the more effective for making micromass pellet cultures, a gene expression analysis between micromass cell cultures obtained with and without centrifugation was performed.

From the graphs, it is clear that no method is substantially better when compared to the other one. It must be noted that the expression of the genes tested thus far, is higher in micromass cultures obtained with centrifugation, although it is statistically significant only for SOX9 and COMP genes overexpressed (p-value < 0.05), with the singular exception of TGFβ1. Given the gene expressions and the simpler way in which micromass cell cultures are obtained when made with centrifugation, this method was subsequently employed.

Chapter I

Appendix

Chondroinduction of chondrocyte micromass culture with stanozolol

Abstract

Aim: The purpose of this study is to evaluate the effect of a 100 nM stanozolol treatment in an equine chondrocyte micromass cell culture. **Materials and methods:** Micromass chondrocyte cultures were established, and, after 14 days of treatment, gene expression was assessed through real-time PCR. There were two conditions, with the only difference being 100 nM stanozolol treatment. In both cases, the medium was a chondrogenesis permissive medium. The genes whose expression variation was assessed included the following: SOX9, CD44, COL2A1, LUM, BGN, COMP, and COL1A1. Statistical evaluation was achieved via t test, considering the results significant for p-value < 0.05. **Results:** The upregulation of COL1A1 and COMP genes in stanozolol treated micromass culture was the only statistically significant difference found. **Discussion:** Due to the fact that the difference between treatments was related to genes codifying for cartilage ECM components, we concluded that stanozolol treatment improved chondrogenesis, even though not as effectively as reported in literature.

Introduction

As seen in the previous experiment, the most effective way to carry out a study aimed at inducing chondrogenesis is the micromass culture of chondrocytes produced by centrifugation. As previously underlined, this culture method tends to simulate the adult cartilage environment [9] and promote chondrogenesis via CD44 stimulation [13][14].

Androgens have anabolic potential, and the main male androgen is testosterone, which can be converted into dihydrotestosterone (DHT), its main active form, by 5α-reductase, or, in estradiol by aromatase. Among the synthetic steroids, stanozolol is of particular interest because of its ability to improve bone and cartilage regeneration, as has already been described in the literature [29]. Stanozolol, a synthetic derivative of DHT, is a non-aromatizable steroid; it has already been used with success for the treatment of pathologies such as rheumatoid arthritis, Reynaud's phenomenon, lipodermatosclerosis, and arteriosclerosis [30]. Within 12 days of treatment, this steroid promotes the induction of genes involved in osteogenic differentiation in a dose-dependent manner, aside from inducing calcified matrix deposition [29]. Stanozolol induces chondrocyte proliferation *in vitro* in a dose and time-dependent manner, reaching its peak after two days of treatment, although it seems to suppress it after five days [31], and, furthermore, it induces osteoblast proliferation, collagen synthesis, and IGF1 secretion. In rats, this molecule induces growth plate chondrocyte proliferation, inhibits NO production, and stimulates the synthesis of collagen and other cartilage ECM components. In horses, the stanozolol treatment reduces the clinical signs of pain in cases of articular issues, both in the acute and chronic phases, and improves the response to flexion [32].

Despite all the knowledge regarding the positive effects of stanozolol, its pathway of action is still unknown. This steroid seems to have an effect that is more anabolic than androgenic compared with testosterone. Furthermore, it seems to not interact actively with the androgen receptor (AR), but, depending on which source is considered, it is believed to interact with estrogen receptor α (ERα) (thus making it more similar to 17β-estradiol in terms of receptor affinity) [31], or it is believed to interact with progesterone receptor (PR) [30]. The latter source seems to indicate that its effect is mediated not only by PR but also by this steroid interaction with another unknown receptor that is responsible for PGE_2 induction. This unidentified receptor, which does not appear to be one of the classic androgen (AR), estrogen (ERα, ERβ), or glucocorticoid (GR)

receptors, seems to be present in skin fibroblasts but not in synovial fibroblasts (in rheumatoid disease), since in the latter cell type, the PGE_2 induction appears not to be present [30].

This experiment aims to verify the ability of stanozolol treatment in inducing chondrogenic differentiation in a micromass culture of chondrocytes; this capability will be assessed by gene expression analysis through real-time PCR.

Materials and methods

Cell culture

Since this experiment is an appendix of the previous one, the cell model used was equine primary chondrocyte cell line. Cells were harvested according to the aforementioned protocol. Micromass cell culture was used to discover the effect of stanozolol in the chondrogenesis process. Considering the results shown in the previous experiment, micromasses were formed through centrifugation of 500,000 chondrocytes/micromass at 500 g for 5 minutes in 5 ml conical tubes. The micromass formation, during the first 48 h of pellet culture, was accomplished leaving the cells in the aforementioned "minimal medium", composed of DMEM high glucose with the addition of 2 mM glutamine, 1% ITS+, and 1X penicillin/streptomycin. During the whole experiment, micromass cultures stayed in a cell incubator at 37° C, as before, the composition of the cell incubator air was not very significant since, not only were the conical tubes airtight but, after every media change, before closing the tube stopper, air was exchanged with a gas mixture composed of 94% N_2, 5% CO_2, and 1% O_2. The media used were composed as follows: the control medium, which was a chondrogenesis permissive medium, was made out of DMEM high glucose supplemented with 2 mM glutamine, 1% ITS+, 40 µg/ml proline, 200 µM ascorbate, 100 µg/ml sodium pyruvate, 10 ng/ml TGFβ1, and 1X penicillin/streptomycin, while the stanozolol containing medium was made in an identical manner but with the obvious addition of a 100 nM stanozolol. Media were changed every 2 to 3 days and the treatments lasted for 14 days. In order to address that which was already evident, dexamethasone was not put in any of the used media. Although this molecule is a common component of

chondrogenic differentiation medium, its effect could have scrambled the stanozolol pure effect. Stanozolol powder was resuspended in absolute ethanol at a concentration of 1 mM.

Gene expression analysis

The protocols and the kits used for micromass culture, RNA extraction, retrotranscription, and real-time PCR, were the same as in the previous experiment.

Primers used in this work are listed in the table below:

Gene name	Forward primer	Reverse primer
RPL15	CTCTTTCCTTTCCGTCTG	GAGAAGAAATCGCATCAC
SOX9	CTCTGGAGACTGCTGAAC	GTTCTTCACCGACTTCCT
COL2A1	CTGGTGATGATGGTGAAG	GTAACCTCTGTGACCTTTG
COMP	GACCAGAAGGACACAGAT	TATACCATCGCCATCACT
CD44	AAGACTCCCACTACCAAA	TCCATTGCCATTGTTGAT
COL1A1	GCAAGAACGGAGATGATG	AACCACTGAAACCTCTGT
LUM	CTGGAGGTCAATGAACTTG	CATATCAGGTGGCAGACTA
BGN	TCTACTTCGCTACTCCAA	GCTGATGCCATTGTAGTA

Table 3. *Table indicating primers that were used to perform real-time PCR assay*

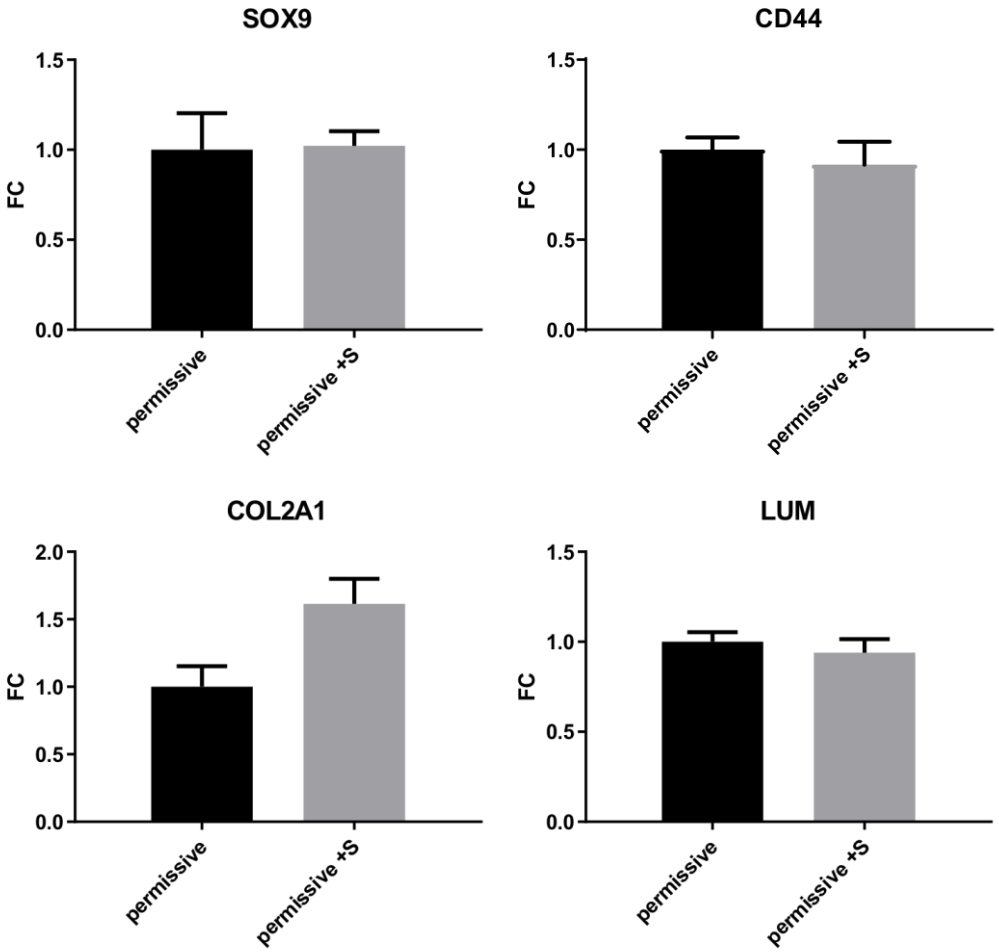
Statistical analysis

Again, in this experiment, PCR relative quantification was performed using the Pfaffl formula to assess the variation of the gene expression ratio. This formula, as opposed to the classical $2^{-\Delta\Delta Ct}$ method, takes into account primers amplification efficiency [26]. P-value was assessed via t test, defining statistical significance at p-value < 0.05, with GraphPad Prism 6 software.

Results

Gene expression analysis

All the gene expressions were normalized with the housekeeping gene RPL15. The real-time PCR results are shown in the following figure:



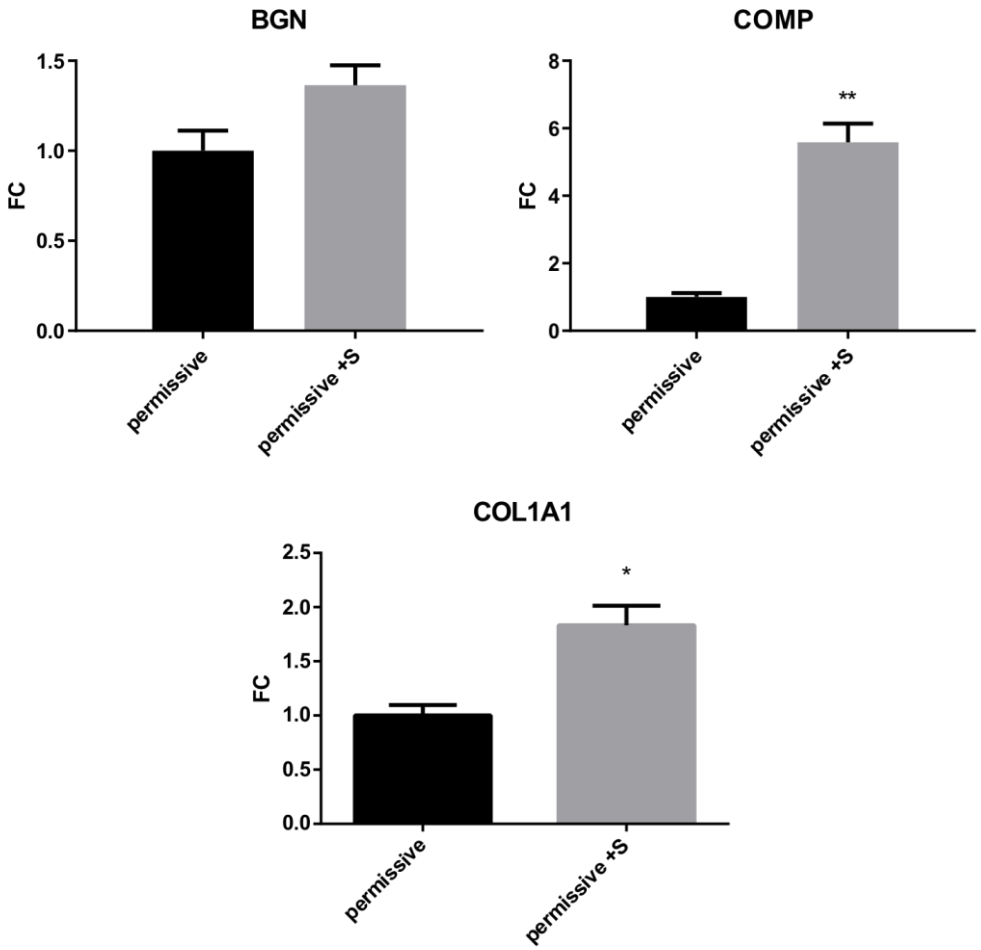


Figure 4. *These graphs show the fold change expression variations (FC) of SOX9, CD44, COL2A1, LUM, BGN, COMP, and COL1A1 genes for the conditions used; gene expression was assessed with real-time PCR and analysed with Pfaffl formula [26]; p-value was considered significant for p-value < 0.05 (* p-value ≤ 0.05,** p-value ≤ 0.01, *** p-value ≤ 0.001). It was calculated via t test performed with GraphPad 6.0. Names on the x axis indicate the two conditions used, both with a chondrogenesis permissive medium, and one of which, shown on the right, included the addition of 100 nM stanozolol. The control condition was the medium condition without stanozolol.*

The genes analysed are all chondrogenesis markers, and the proteins translated from these genes' transcripts are part of the cartilage ECM, with the exceptions of SOX9 and CD44. COMP and COL1A1 are both significantly overexpressed in the micromass culture whose medium included the addition of 100 nM stanozolol (COMP fold change = 5.581617, SD = 0.556859, p-value = 0.0076; COL1A1 fold change = 1.833283, SD = 0.179523, p-value = 0.0287), while the COL2A1 and BGN overexpressions would have been significant, once again, if the confidence level was set on 90% instead of 95 (COL2A1 fold change =

1.614530, SD = 0.184695, p-value = 0.0682; BGN fold change = 1.364283, SD = 0.110136, p-value = 0.0818). COL10A1 gene expression was not detected.

Discussion

There are only three new genes whose expressions were not previously analysed in chapter 1, and they are the following:

COL1A1: Type I collagen α1 chain - although it is bone matrix main component, collagen I can also be found in other connective tissues, such as tendon, derma, and cartilage. Although collagen I is heavily involved in endochondral ossification, it is also expressed during cartilage ECM formation [27].

BGN: Biglycan - the protein codified from this gene has different functions, the main one being the collagen fibril assembly; it is also involved in regeneration patterns, muscle development, and bone growth.

LUM: Lumican - this gene encodes a protein that belongs to the same family of BGN; it regulates tissue repair and collagen fibril organization.

Due to lack of regenerative potential that characterizes cartilage, both the establishment of a method aimed to produce cartilage *in vitro* and the discovery of molecules able to induce cartilage regeneration are of paramount importance. Stanozolol, even acting through an unknown pathway, has shown regenerative potential, and its effect was tested during this experiment. This study was carried out using a differentiation permissive medium without dexamethasone. As shown in the first chapter, micromass cell culture method not only induces chondrogenesis but also serves to create an environment that simulates the one present in adult cartilage tissue.

Results show that, at 14 days of stanozolol treatment, this molecule is capable of augmenting the gene expression of ECM components. Although we assessed the statistically significant induction of COMP and COL1A1 genes along with the increase of COL2A1 and BGN gene expressions, stanozolol effect resulted not so evident in inducing chondrogenesis. It is important to underline that although the results seem not to be consistent with the stanozolol effectiveness reported in literature [32], the data differ probably due to the

different complexity of the chondrogenic milieu. Furthermore the ratio between COL2A1 and COL1A1 gene expressions (0.89 in permissive+S condition vs 1.00 in permissive condition) can indicate the likely deposition of fibrocartilage following stanozolol treatment *in vitro*.

Chapter II

The effect of stanozolol and hyaluronic acid hydrogel co-treatment in the osteoinduction of HOB-01-C1 cell model

Abstract

Aim: This study assesses the ability of stanozolol and stanozolol co-treatment with hyaluronic acid hydrogel to induce osteogenesis in HOB-01-C1 cell model. **Materials and methods:** HOB-01-C1 cell model is the preosteocyte-like cell model used in this experiment. The osteogenesis permissive medium, with or without 100 nM stanozolol, was used as a control medium. All conditions were performed with or without treatment with a 17 mg/ml hyaluronic acid hydrogel. The experiment lasted for 21 days, and both calcified matrix deposition and gene expression were assessed, the latter with real-time PCR assay and the former with microscopic observation of the culture plate, thanks to the addition of 2 μM Calcein Green to the culture media. The genes evaluated in their expression ratios were: RUNX2, COL1A1, SOST, BMP2, PHEX, and VDR. Statistical evaluation was performed via one-way ANOVA, followed by Dunnett's multiple comparisons test, considering the results significant for p-value < 0.05. **Results:** Both Calcein Green assay and real-time PCR assay showed related results: osteogenesis augmented in the medium containing stanozolol, and it was even more marked when the hyaluronic acid co-treatment was present. **Discussion:** Stanozolol managed to induce a more efficient osteogenesis, and its effect was even more marked when accompanied by CD44 stimulation due to hyaluronic acid hydrogel co-treatment.

Introduction

Bone tissue, as well as cartilage tissue, is a connective tissue whose cell lineage arises from MSCs differentiation. During embryonic development, bone matrix is formed from the endochondral ossification phenomenon, which involves chondrocytes that become hypertrophic after the anlagen formation. The anlagen was a result of the mesenchymal condensation event that takes place when MSCs aggregate. The endochondral ossification event (with COLX being the specific marker of the hypertrophic chondrocyte) is the phenomenon from which the whole skeleton arises, with the exception of the clavicles and skull. In the skull, endochondral ossification leads to the formation of the bottom and posterior parts, while the rest of its structure is produced by intramembranous ossification led directly by osteoblasts.

During embryonic development, bone takes the place of mineralized cartilage matrix. It is characterized by a mineralized ECM predominantly made out of elements, such as type I collagen (COLI) and osteonectin (ON), as far as its organic portion is concerned, while its mineral component includes hydroxyapatite crystals that originate from amorphous calcium phosphate. ECM is produced by a specific cell type, osteoblasts, which is part of the delimitating structure that can be seen as a sort of epithelium; this structure separates, for example, the bone from its marrow section. During matrix production, osteoblasts that get enveloped in it terminally differentiate in osteocytes. Osteocytes are the main constituent of the bone cell population and are kept apart from each other in cavities called lacunae. The connections osteocytes make among each other, and with the osteoblasts of the superficial layer, are cytoplasmic extensions placed in structures called canaliculi [33][34]. Bone tissue performs different functions, such as serving as a calcium reservoir and giving structural support.

The cell model called HOB-01-C1 is thought to be a preosteocyte/osteocyte-like cell model obtained from trabecular bone cells [35]. As stated in their patent (US 2011/0020348 A1 published on January 27, 2011), following the transformation with Large T antigen tsA 209 (a thermosensitive variant of the original SV40 Large T antigen), this cell line replication ability is granted at a permissive temperature of 34° C, with a doubling time of 5 to 6 days; whereas, at a temperature equal or superior to 37° C, replication capability is inhibited. This cell model, thought to be incapable of forming a monolayer, expresses the CD44 hematopoietic isoform, as is consistent with its mesenchymal origin [36].

Despite this cell line characterization, HOB-01-C1 shows peculiarities such as the production of chemokines and cytokines (MCP-1 and IL-6) after treatment with IL-1β. This behaviour seems to be typical of cells with an osteoblastic nature and seems not to be typical of osteocytes dwelling in the mineralized bone matrix [36]. Furthermore, after a critical number of cell passages (between 15 and 20), this cell model seems propense to go toward modifications that makes it phenotypically different and that turn it into a slightly different cell model called HOB-01-C1-PS-09. This "–PS-09" variant, despite having a preosteocytic phenotype, shows an improved replication ability, doubling its cell number every 2 to 3 days, and, unlike the "not-PS-09" cell line, does not express OC in response to the treatment with vitamin D3 at 34° C. At 37° C, HOB-01-C1-PS-09 increases its ALPL expression about 4 fold [36].

To conclude that the phenotype of this cell line is not highly stable or even definable with certainty, especially when going through a certain number of passages, we must consider what was previously said regarding the HOB-01-C1 response to IL-1β treatment, while keeping in mind what was mentioned about the modifications that originated in the model which have the "-PS-09" suffix. In addition, this cell line seems not to express SOST [35], a terminal differentiation marker expressed in other cell lines, such as SaOS2 cell model, a typical mature-osteoblastic model [37] that, because of its differentiation degree, has to be theoretically less differentiated when compared to the HOB-01-C1 cell line. This phenotype instability is the reason why the HOB-01-C1 cells used in this specific study show some differences when compared to the same cell type described in the literature (or in the specific patent): first of all, they showed a different doubling time at non-permissive temperature (calculated on 144 hours of culture) of about 35 hours at 37° C and 36.5 hours at 39° C; secondly, they managed to form a monolayer due to the flask cell amplification at 37° C (as shown below in Figure 5); and, lastly, regarding gene expression, this cell line still expresses osteogenic markers such as RUNX2, ALPL, and ON.

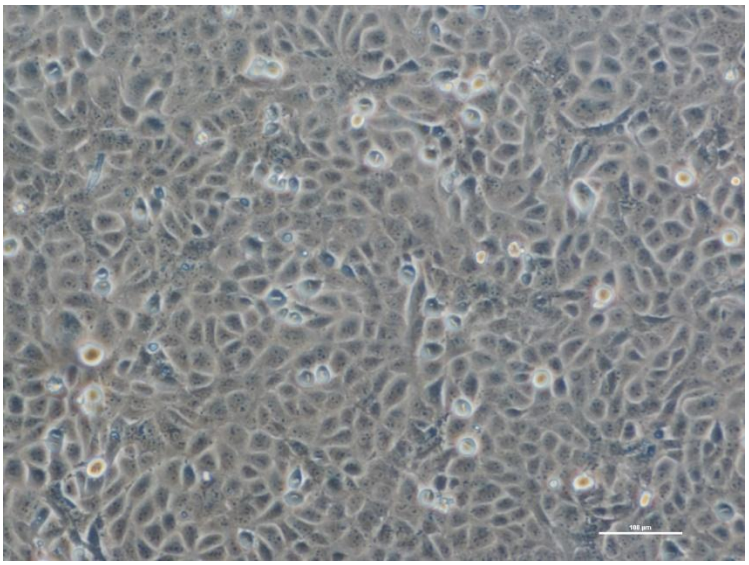


Figure 5. *10X magnification photo of a HOB-01-C1 monolayer grown in a 75 cm^2 flask at 37° C; cells were kept in a DMEM low glucose medium with 10% FBS, 2 mM glutamine, and 1X penicillin/streptomycin.*

The HOB-01-C1 cell line expresses CD44 which, following its stimulation, through interaction with, for example, hyaluronic acid (HA) [13], supports bone mineralization through BMP2 induction [14]. BMP2 mediates ALPL activation, increasing RUNX2 nuclear accumulation and promoting calcified nodules formation [15].

This experiment seeks to evaluate the ability of stanozolol treatment to induce mineralization and differentiation of HOB-01-C1 cell line, with or without HA hydrogel co-treatment. The evaluation of the effects of these treatments will be carried out both quantitatively, with gene expression evaluation, through real-time PCR, and qualitatively, with calcified matrix deposition assessment. This last data will be obtained with the direct observation, at specific time points, of the calcified matrix deposed, which was labelled with the introduction of 2 μM Calcein Green in the culture media, according to methods previously described in the literature [38].

Materials and methods

Cell culture

HOB-01-C1 cells were seeded in a 6 wells plate at 200,000 cells/cm^2. After 24h rest, in a DMEM low glucose medium with 10% FBS, 2 mM glutamine, and 1X penicillin/streptomycin, experimental conditions were applied. In this experiment, three different media were used, all three both with and without the use of an additional variable, which, in this case, was the presence of a hyaluronic acid hydrogel. The media used, were composed as follows: the control medium was a DMEM low glucose medium with 10% FBS, 2 mM glutamine, and 1X penicillin/streptomycin; the permissive medium was a DMEM low glucose medium with 10% FBS, 2 mM glutamine, 40 µg/ml proline, 100 µM ascorbate, 2 mM β-glycerophosphate, and 1X penicillin/streptomycin, and the differentiation medium was the same as the permissive one, with the addition of 100 nM stanozolol. The β-glycerophosphate concentration was chosen taking into account that a concentration of this factor superior to 2 mM tends to induce the formation of dystrophic or non-apatitic mineralization nodules, even in cells incapable of osteogenic differentiation [39]. Stanozolol solution was obtained in the manner described in the chapter 1 appendix. The hydrogel laid upon the cell monolayer was a 17 mg/ml HA hydrogel; it was obtained by the overnight action of a rotating magnet, aimed at mixing 170 mg of HA with 10 ml of PBS. Once obtained, the hydrogel was sterilized in an autoclave with a sterilization time of 17 minutes at 121° C. Media were changed every 2 to 3 days, and, after every media change, Calcein Green was added at each well in a 2 µM final concentration. The experiment lasted for 14 days; the plate was kept in a cell incubator at 39° C and 5% CO_2.

Real time Calcein Green assay

Before every media change the mineralized matrix, labelling with Calcein Green was assessed through qualitative observation with a fluorescent microscope (Nikon Eclipse TE300 inverted microscope), with the light emitted by a super high pressure mercury lamp. Mineralization photos were taken at a 20X magnification. Since the control medium condition without HA hydrogel never showed the deposition of calcified nodules, photos of this specific condition are not shown in the results.

Gene expression analysis

RNA extraction was performed at the end of the treatment, just after the last observation of the Calcein Green labelling. First, all the media and the hydrogel layers (where present) were removed carefully, in order not to detach the cells. Afterwards, Trizol was put into wells, and after few seconds of pipetting, was taken and put in vials; 0.2 ml of chloroform were added for every ml of Trizol used. The tubes were shaken for 15 seconds and put to rest for 3 minutes at room temperature, and then, after the solution became biphasic, they were centrifuged for 15 minutes at 12,000 g at 4° C. In the next step, the upper aqueous phase, containing RNA, was removed, and 0.5 ml of 100% isopropanol was added for every ml of Trizol used; the solution obtained was laid to rest at room temperature for 10 minutes and then was centrifuged at 12,000 g for 10 minutes at 4° C. At the end of this last centrifugation, RNA pellet was maintained, discarding the supernatant, and then 1 ml of 75% ethanol was added for each ml of Trizol used. The resulting RNA solution went through another centrifugation for 15 minutes at 12,000 at 4° C; then, supernatant was discarded, and the RNA pellet was left to dry in open air. RNA was suspended in 50 µl of RNase free H_2O and was mixed with 100 µl of Monarch RNA Cleanup Kit RNA Cleanup Buffer, and, afterwards, we followed the steps and used the solutions belonging to this same purification kit, everything according to the manufacturer protocol; in the end, the purified RNA was suspended in 50 µl of RNase free H_2O.

RNA transcription into cDNA and real-time PCR were all carried out according to the methods previously described in chapter 1.

Primers used in this work are listed in the table below:

Gene name	Forward primer	Reverse primer
RPL15	GCAGCCATCAGGTAAGCCAAG	AGCGGACCCTCAGAAGAAAGC
RUNX2	CCAGGCAGGCACAGTCTTC	GTCAGAGGTGGCAGTGTCATC
COL1A1	GTCGAGGGCCAAGACGAAG	CAGATCACGTCATCGCACAAC
SOST	TGTCTCGTCTGCCTGCTG	GTTCATGGTCTTGTTGTTCTCC
BMP2	GTATCGCAGGCACTCAGGTC	GGTTGTTTTCCCACTCGTTTC
PHEX	AGGCATCACATTCACCAACAAC	GCACCATTGACCCTAAACTGAG
VDR	GAAGCGGAAGGAGGAGGA	TGGGAGTGTGTCTGGAGTTG

Table 4. *Table indicating primers that were used to perform real-time PCR assay*

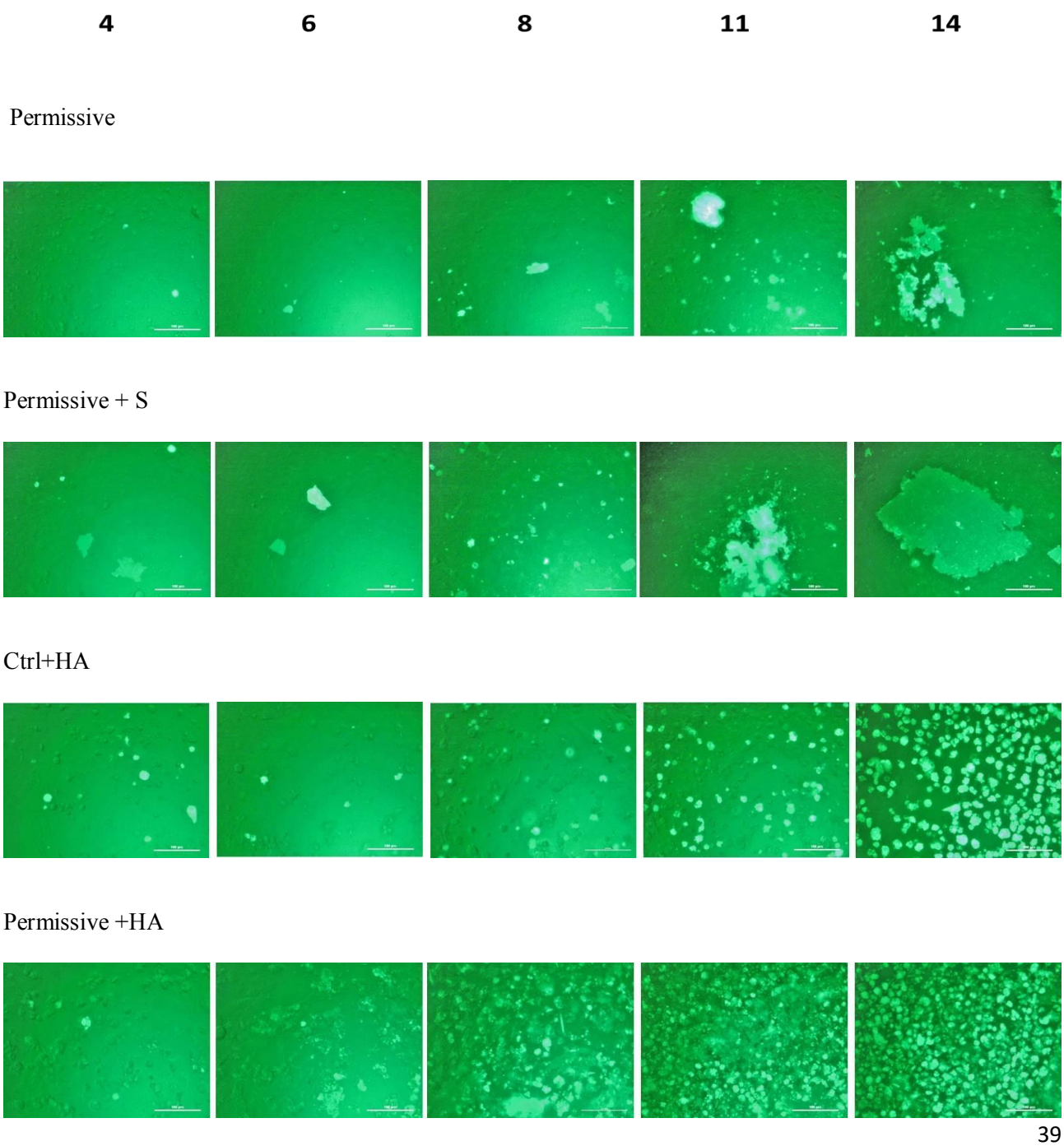
Statistical analysis

Once again, PCR relative quantification was performed using the Pfaffl formula to assess the variation of the gene expression ratio. This formula, unlike the classical $2^{-\Delta\Delta Ct}$ method, takes into account the' amplification efficiency of the primers [26]. P-value was assessed through one-way ANOVA, followed by Dunnett's multiple comparisons test, defining statistical significance at p-value < 0.05, with GraphPad Prism 6 software.

Results

Calcein green assay

As previously stated, control medium condition without HA (ctrl) never showed calcified matrix deposition, and, therefore, there is no image of this specific condition in the figure below. Photos were captured at 4, 6, 8, 11, and 14 days of treatment.



Permissive + S +HA

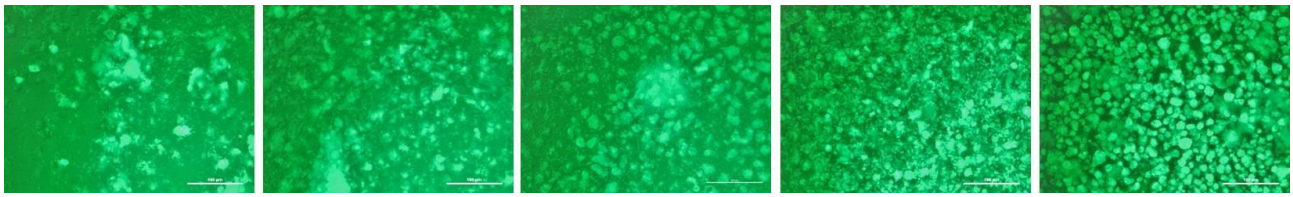


Figure 6. *These photos were captured before every media change, at 4, 6, 8, 11, and 14 days of treatment, as written above each figure. Names on top of each row of photos indicate the conditions used; HA indicates 17 mg/ml hyaluronic acid hydrogel, while S indicates 100 nM stanozolol; the control condition (ctrl) is not present because it never showed calcified matrix deposition. Images were captured at 20X magnification with a fluorescent microscope (Nikon Eclipse TE300 inverted microscope) with the light emitted by a super high pressure mercury lamp.*

It appears evident how calcified nodules are more present in the conditions containing HA; for that reason, ctrl condition showed calcifications only with the simultaneous HA treatment, and this shows how CD44 stimulation is osteoinductive. Furthermore, stanozolol treatment seems to increase the number (with HA hydrogel) or the dimension (without HA hydrogel) of the calcified nodules.

Gene expression analysis

Gene expression was assessed using RPL15 as a housekeeping gene. The graphs of the fold changes are shown in the following figure:

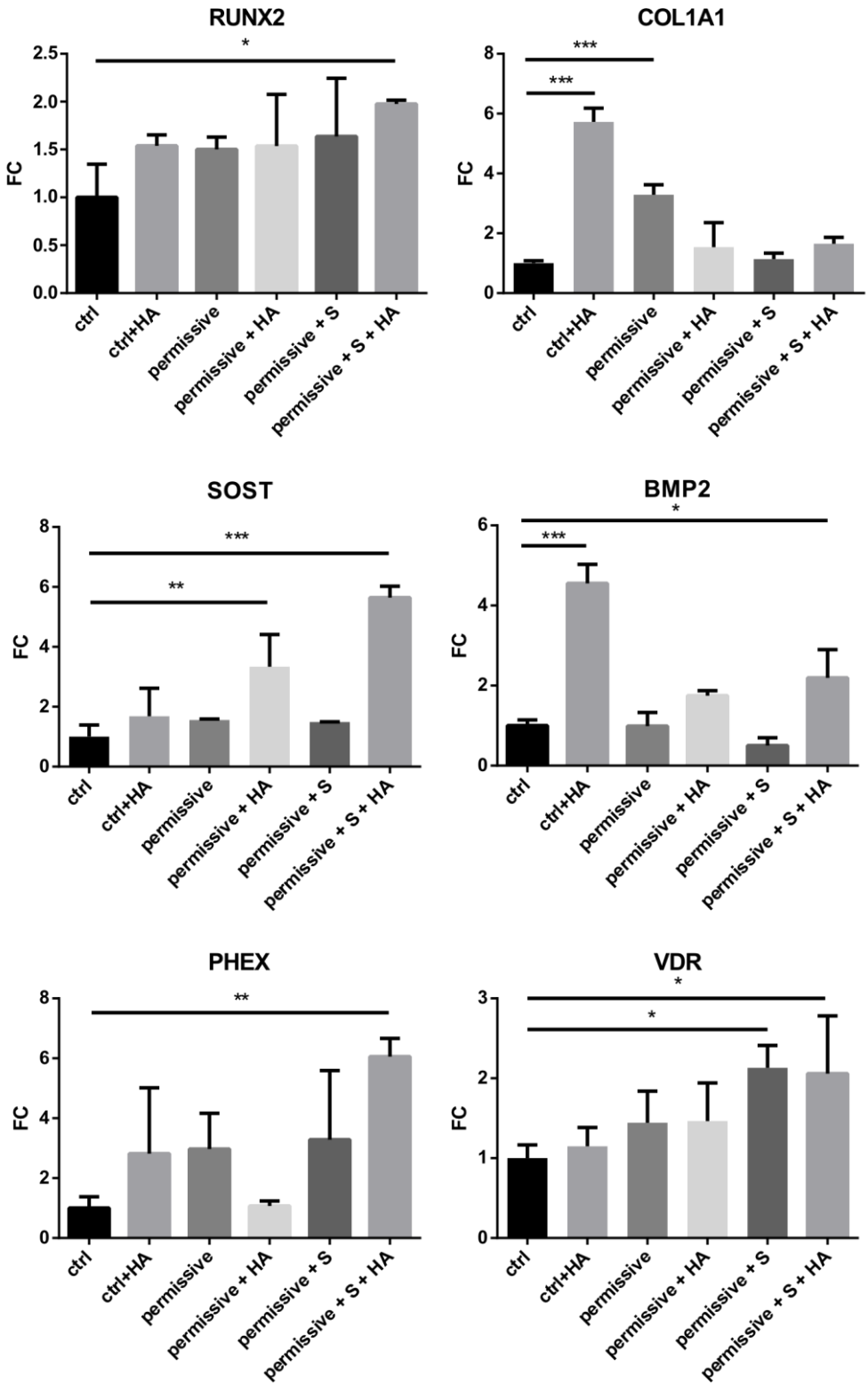


Figure 7. *These graphs show the fold change expression variations (FC) of RUNX2, COL1A1, SOST, BMP2, PHEX, and VDR genes for the various conditions used; gene expression was assessed with real-time PCR and analysed with Pfaffl formula [26]; p-value was considered significant for p-value < 0.05 (* p-value ≤ 0.05,** p-value ≤ 0.01, *** p-value ≤ 0.001). It was calculated via one-way ANOVA, followed by Dunnett's multiple comparisons test performed with GraphPad 6.0. Names on the x axis indicate the conditions used; HA indicates 17 mg/ml hyaluronic acid hydrogel, and S indicates 100 nM stanozolol; the control condition is on the left side of the graph, indicated as ctrl.*

The graphs show trends consistent with what was observed after the Calcein Green assay. COL1A1 overexpression is significant only in the ctrl+HA and the permissive conditions (ctrl+HA fold change = 5.723837, SD = 0.456849, p-value < 0.0001; permissive fold change = 3.290501, SD = 0.330115, p-value = 0.0001); this gene is expressed only in the first stages of the osteogenesis and then its expression fades away. Furthermore, the SOST fold change variation is consistent with what was stated in the previous section about the effects of HA and stanozolol, being progressively more expressed in the HA conditions along with the difference in treatment. SOST increases its expression with the presence of permissive medium, and even more so, with the addition of 100 nM stanozolol (ctrl+HA fold change = 1.684521, SD = 0.931792, p-value = 0.5510; permissive+HA fold change = 3.335908, SD = 1.075033, p-value = 0.0026; permissive+S+HA fold change = 5.645730, SD = 0.380671, p-value < 0.0001). RUNX2 and VDR basically show the same gene expression trends for the same conditions, and, lastly, BMP2 shows an overexpression in those conditions in which there is HA hydrogel, which is in line with previous knowledge that CD44 acts through this gene (ctrl+HA fold change = 4.552157, SD = 0.477099, p-value < 0.0001; permissive+HA fold change = 1.748586, SD = 0.124626, p-value = 0.1281; permissive+S+HA fold change = 2.193857, SD 0.703078, p-value = 0.0111).

Discussion

Since bone regeneration is a slow and complicated process, it is important to understand how it can be improved with the help of expedients intended to support and promote the actual differentiation pathway. In this study, two treatments were tested: cell growth in a HA hydrogel and 100 nM stanozolol treatment.

Only four new genes whose expression was not previously mentioned in chapter 1 were analysed:

RUNX2 - also called CBFA1, is the osteogenesis master switch; it is a transcription factor essential for osteoblastic differentiation, skeletal morphogenesis, and for both endochondral and intramembranous ossification processes.

SOST: Sclerostin is a bone growth inhibitor and osteoid marker of terminal differentiation; Albert-Shönberg disease, also called osteopetrosis, is an example of a disease caused by this gene's loss of function.

VDR: Calcitriol (the active form of vitamin D3) receptor is a bone differentiation marker; vitamin D3 is involved in mineral metabolism.

PHEX: Phosphate regulating endopeptidase homolog X-linked; the protein encoded by this gene is putatively involved in bone and dentin mineralization processes.

Both Calcein Green and real-time PCR assays gave results that are in accordance. It can be concluded that CD44 stimulation by HA manages to induce bone matrix deposition even without the proper medium (in the ctrl+HA condition), and, as seen in other studies, stanozolol managed to induce osteogenesis, as well, although to a lesser extent than the hydrogel. According to RUNX2, SOST or PHEX gene expressions, stanozolol did not appear to have as great an effect when unaccompanied by the HA hydrogel co-treatment. It is evident that HA and stanozolol together assumed a synergy that is potentially useful in bone regeneration. Comparing these results with the ones described in the chapter 1 appendix, stanozolol seems to need some kind of kickstart action in order to induce an effective differentiation: this priming appears to involve BMP2 action which, in turn, intervened following CD44 stimulation.

It can be concluded that the relatively low effect of stanozolol on this specific cell model can be effectively augmented if accompanied with a co-stimulation with HA, which is able to promote bone regeneration through BMP2 induction. The combination of these factors can be exploited to improve the bone regeneration process.

Chapter III

The identification of the stanozolol target receptor in mesenchymal stem cell model

Abstract

Aim: The objective of this study was to assess which receptor is or receptors are the ones with which stanozolol interacts in order to achieve its effect. **Materials and methods:** In the two stages of this experiment, mesenchymal stem cells immortalized with hTERT were the cell line used. Both stages were carried out with micromass culturing method. In the first stage, two differentiation permissive media were compared to understand which gene can be used as a stanozolol treatment marker; in the second stage, different receptor inhibitors (MPP, flutamide, PHTPP, and mifepristone) were used along with stanozolol to assess which receptor is the one that interacts with the steroid. In both stages, gene expression was evaluated through real-time PCR. In the second stage, western blot assay was also performed to analyse which PKC isoforms were activated by phosphorylation and how much, in the various conditions. Both stages of the treatments lasted for 12 days. The genes analysed in the first stage were as follows: COL2A1, DCN, MATN1, and MATN3; the genes analysed in the second stage were MATN1, MATN3, and DCN. In the first stage, statistical evaluation was achieved via t test, while in the second one, it was performed with one-way ANOVA, followed by Dunnett's multiple comparisons test. In both cases, results were considered significant for $p\text{-value} < 0.05$. **Results:** As far as the first stage's gene expression confrontations are concerned, MATN1 and MATN3 were the two genes whose expressions' fold changes were so significant as to cause them to be the genes chosen as treatment markers during the experiment's second stage. In the second stage, it was highlighted that mifepristone, a progesterone receptor inhibitor, blocked stanozolol's effect; western blot showed the same result, indicating that the only activated PKC isoform was PKCε. **Discussion:** Stanozolol manages to induce mesenchymal stem cell differentiation toward a pathway leading to the formation of interzone's anlagen chondrocytes. This absence of steroid demonstrates how differentiation tends to go towards the development of anlagen chondrocytes that will culminate in undertaking endochondral ossification. Stanozolol interacts with a progesterone receptor, but there is evidence suggesting that it also interacts with another unknown receptor, which is responsible for the induction of PGE_2 biosynthesis as some of its effects seems to involve this cytokine.

Introduction

Bone and cartilage cell lineages stem from differentiation processes borne by MSCs. Stem cells, in general, are cells endowed with specific characteristics such as the capabilities to self-renew, to go toward asymmetric divisions, or to differentiate into different cell lineages. Stem cells can be divided into categories based on their differentiation potential (totipotent, pluripotent, multipotent, oligopotent, and unipotent) or their origin (embryonic, induced pluripotent, mesenchymal, etc).

MSCs are adult multipotent stem cells that can be found in different places like bone marrow, adipose tissue, endometrium, peripheral blood, salivary glands, and synovial fluid [40]. The first MSCs to be isolated were derived from bone marrow and were described for the first time in the 1970s. Possessing a fibroblastoid shape, they were initially called colony forming unit-fibroblasts (CFU-F) [41]. During the next decade, these cells, characterized by a relative heterogeneity, were found to be part of the stroma. It was demonstrated that they build up a niche and were capable of giving structural and functional support to haematopoiesis and to the stem cells involved in this phenomenon [41][42]. Nowadays, the International Society for Cellular Therapy identifies as MSCs, cells that show the capability to at least attach to plastic, express CD73, CD90, and CD105 markers (MSCs nevertheless also express other markers such as CD73 and CD44 [43]), and, at the same time, lack the expression of CD14, CD34, CD45, and HLA-DR (MSCs seem to also lack the expression of other markers, such as CD3 and CD19) [42]. These cells have to show the capability to differentiate, *in vitro*, into adipose, bone, and cartilage cell lineages [40][41].

Although there are still doubts about their actual staminal nature (sometimes these cells are indicated as stromal), and, beside the fact that there is a need to get a greater number of markers for a more specific identification, MSCs are largely studied and utilized for their differentiation ability and still remain cells with huge potential in cell therapy aimed at tissue regeneration [42].

During embryonic development, MSCs go toward an aggregation phenomenon that is called mesenchymal condensation and that leads to the formation of a cartilage structure called anlagen. The anlagen leads to the formation of the axial skeleton and the limbs. In the anlagen, the MSCs go toward a chondrogenic differentiation that will lead to the formation of the very bone or the joint; in the first case, chondrocytes will

terminally differentiate through endochondral ossification. These phenomena are due to the effect of different factors, such as FGF, TGFβ, and BMP2, and are led by the so-called master switches for the bone and cartilage differentiation processes, RUNX2 and SOX9 respectively [2]. The alternative induction of the differentiation patterns toward the formation of chondrocytes or chondrocytes that will initiate endochondral ossification seems to be guided by the variation in the TGFβ/BMP2 ratio along the length of the anlagen itself [44], other than being characterized by the expression of specific markers such as MATN1. MATN1 is specifically expressed in the anlagen cells that will be involved in the bone formation, while the cells that will form the joint, the interzone ones, seem to lack its expression [45].

As seen in the previous experiments, stanozolol's ability to augment differentiation is particularly evident when its presence in the medium is accompanied by a co-stimulation of the CD44 receptor. This double stimulation was present in both micromass cell culture and the HOB-01-C1 cell line culture; in the latter case, this was true only when the HA hydrogel was laid upon the cell monolayer at the beginning of the treatment.

Considering the mesenchymal common origin of the cells used in the previous experiments and the need to stimulate CD44 to get a more evident effect of the stanozolol treatment, this work is aimed towards understanding which receptor is the one with which the steroid actually interacts, between AR, ERα, ERβ, and PR. This study was divided in two stages and was carried out using micromass cell culture formed by MSCs immortalized with hTERT (human telomerase reverse transcriptase):

- In the first stage, the effect of the stanozolol treatment was assessed by studying, with real-time PCR, which gene was more influenced in its expression by the treatment itself;

- In the second stage, the inhibition of the various receptors mentioned above was evaluated to understand which one is responsible for stanozolol's effect. This evaluation was executed by assessing, through real-time PCR, the gene expression of the genes chosen after the completion of the first stage. The data obtained was supported with protein activation analysis with western blot assay.

Materials and methods

Cell culture

Immortalized MSCs are the cell line used in this experiment. These cells are called hTERT MSCs because their immortalization is due to the introduction of hTERT, the factor that grants this cell the capability not to senesce after various steps, without inducing tumorigenic changes in the cell genotype. Micromass cell cultures were made out of 250,000 cells/micromass. After a few days' amplification in a 75 cm^2 flask in a DMEM/F12 medium with 10% FBS, 2 mM glutamine, and 1X penicillin/streptomycin, cells were detached and were resuspendend in a minimal medium composed by DMEM high glucose with 1% ITS+, 2 mM glutamine, and 1X penicillin/streptomycin. The following centrifugation, at 500 g for 5 minutes, led to the formation of cell pellets that were laid to rest for 48 h, until the completion of micromass formation. Micromass cell cultures were made, once again, in airtight 5 ml conical tubes (rendering the outer culture air composition irrelevant), and, after their formation, the minimal medium was switched with the experimental conditions: the control medium was a differentiation promoting DMEM high glucose medium with 2 mM glutamine, 1% ITS+, 40 µg/ml proline, 200 µM ascorbate, 100 µg/ml sodium pyruvate, 10 ng/ml TGFβ3, and 1X penicillin/streptomycin, while the positive control medium was identical with the addition of 100 nM stanozolol. The experiment lasted 12 days, during which cells were kept in a cell incubator at 37° C; media were changed every 2 or 3 days.

Gene expression analysis

RNA extraction was performed with Trizol solution. In short, micromass cultures were placed in Trizol into Eppendorf tubes and homogenized with CAT X120 homogenizer. Afterwards, 0.2 ml of chloroform was added per ml of Trizol used, and, after 15 seconds of shaking and 3 minutes of resting, the aqueous and organic phases were separated by centrifugation at 12,000 g for 15 minutes at 4° C. While the organic phase was used to perform protein extraction, the upper aqueous phase containing the RNA, was mixed with 0.5 ml of 100% isopropanol for every ml of Trizol used. The RNA-isopropanol solution was put to rest for 10 minutes at room temperature and was then centrifuged at 12,000 g for 10 minutes at 4° C. Following the

centrifugation, the pellet was washed with 1 ml 75% ethanol for every ml of Trizol used, and, after a final centrifugation at 12,000 g for 15 minutes at 4° C, supernatant was discarded and RNA was, first, air dried and, then, suspended in 50 µl H_2O. The RNA solution was mixed up with 100 µl of Monarch RNA Cleanup Kit RNA Cleanup Buffer for RNA purification, performed following the steps and using the solutions belonging to the Monarch RNA Cleanup Kit, according to the manufacturer protocol. In the end, the purified RNA was suspended in 50 µl of RNase free H_2O.

RNA transcription into cDNA and real-time PCR were all performed using the methods previously explained in chapter 1.

Primers used in this work are listed in the table below:

Gene name	Forward primer	Reverse primer
RPL15	GCAGCCATCAGGTAAGCCAAG	AGCGGACCCTCAGAAGAAAGC
COL2A1	GCAGCAAGAGCAAGGAGAAG	GTGGACAGCAGGCGTAGG
DCN	TTAGTCCTGGAGCATTTACACCT	GTGCCCAGTTCTATGACAATCA
MATN1	CAGAGCACTACTTCTACAC	GAATTTCACCAGGGACTC
MATN3	GTGAATGCTATGAGGGCTAC	AACAAGTCTTCGTGCTTCC

Table 5. *Table indicating the primers used to perform real-time PCR assay*

Statistical analysis

In this experiment, PCR relative quantification was performed using the Pfaffl formula to assess the variation of the gene expression ratio; this formula, unlike the classical $2^{-\Delta\Delta Ct}$ method, takes into account the

amplification efficiency of the primers, rendering the data more solid [26]. P-value was assessed by t test during the experimental first stage and via one-way ANOVA, followed by Dunnett's multiple comparisons test during the second stage, defining statistical significance at p-value < 0.05, with GraphPad Prism 6 software.

The effect of stanozolol treatment in micromass MSC culture

Results

Gene expression analysis

In every stage, RPL15 was used as the housekeeping gene.

Gene expressions are shown in the figure below:

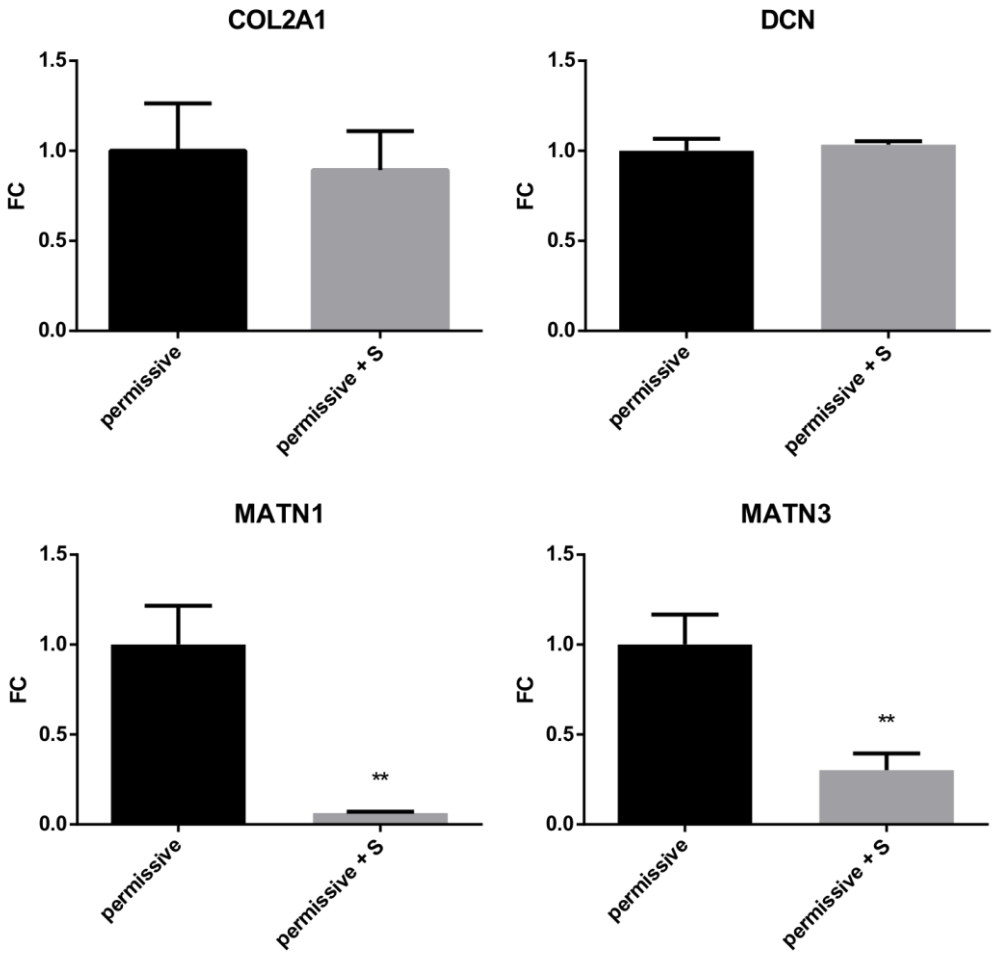


Figure 8. *These graphs show the fold change expression variations (FC) of COL2A1, DCN, MATN1, and MATN3 genes for the conditions used; gene expression was assessed with real-time PCR and analysed with Pfaffl formula [26]; p-value was considered significant for p-value < 0.05 (* p-value ≤ 0.05,** p-value ≤ 0.01, *** p-value ≤ 0.001). It was calculated via t test performed with GraphPad 6.0. Control medium was the permissive one, while the other was identical apart from the addition of 100 nM stanozolol.*

MATN1 and MATN3 are the genes whose expression is mainly affected by 100 nM stanozolol treatment. While the other genes, encoding for ECM proteins, are basically equally expressed, a significant underexpression was detected in MATN1 and MATN3 (MATN1 fold change = 0.06330249, SD = 0.007498, p-value = 0.0017; MATN3 fold change = 0.3020769, SD = 0.092566, p-value = 0.0032).

Discussion

The genes whose expression was analysed and that were not previously cited include:

DCN: Decorin - the protein encoded by this gene plays a role in angiogenesis inhibition and in collagen fibril assembly.

MATN1 and MATN3: Matrilin 1 and 3 - the proteins encoded by these genes are involved in the establishing of the cartilage matrix filamentous network. Although these genes are important in the maintenance of adult cartilage ECM, after the anlagen formation, during embryonic development, $MATN1^-$ interzone MSCs will form articular cartilage, while the $MATN1^+$ MSCs will go toward the formation of transient hypertrophic cartilage that will be replaced by bone [45].

Stanozolol's pathway of action is still unknown. In the literature, there are different and mutually exclusive assessments in which this steroid is said to interact with ERα [31] or with PR and another not identified receptor responsible for the PGE_2 production [30]. Since stanozolol is a synthetic and non-aromatizable derivative of DHT, and given its usefulness in terms of cartilage and bone regeneration, it is important to understand its mechanism of action.

The results show that MSC micromass culture, which creates an environment intended to simulate mesenchymal condensation [12], goes toward articular cartilage formation if treated with stanozolol and undergoes a differentiation pathway that tends to result in bone formation if not subjected to the effect of stanozolol. This is the conclusion that can be drawn from the underexpression of both MATN1 and MATN3 in stanozolol's presence, as anlagen interzone, which will eventually result in joint formation, is formed, in embryonic development, by MSCs that do not express MATN1 [45].

The steroid receptor inhibitors' effect on stanozolol treatment MSC micromass culture

Materials and methods

Materials and methods were previously described.

Cell culture

The cell culture setting of this second stage was the same as the previous one, except for the medium conditions. In addition to the replication of the first stage's medium conditions, there were others, intended to test the stanozolol receptor interaction. The second stage's additional conditions were identical to the first stage's positive control with the addition of a single inhibitor: 1 μM flutamide was in the AR inhibition medium; 10 nM MPP dihydrochloride hydrate (MPP) was in the ERα inhibition medium; 1 μM PHTPP was in the ERβ inhibition medium; and 20 nM mifepristone was in the PR inhibition medium.

Western Blot assay

Protein extraction was performed starting from the organic phase obtained from the conditions of the experiment's second stage. At first, DNA was precipitated through a series of steps involving the addition of 0.3 ml of 100% ethanol for every ml of Trizol used to the organic phase; after mixture and 3 minutes rest at room temperature, DNA was precipitated with a centrifugation at 2,000 g for 5 minutes at 4° C. The phenol-ethanol supernatant was kept for the same protein extraction. 1.5 ml of 100% isopropanol per ml of Trizol

used was added to phenol-ethanol protein solution, and the obtained solution stayed at room temperature for 10 minutes, after which it was centrifuged at 12,000 g for 10 minutes at 4° C. The resulting protein pellet was washed 3 times with 2 ml of a solution of 0.3 guanidine hydrochloride in 95% ethanol per ml of Trizol used; this washing step, which was performed 3 times, consisted in an initial 20 minutes rest at room temperature, followed by centrifugation at 7,500 g for 5 minutes at 4° C. After the conclusion of the washing steps, 2 ml of 100% ethanol was used to suspend the protein, and, after another 20 minutes rest and another centrifuge at 7,500 g for 5 minutes at 4°C, supernatant was discarded and protein pellet was air dried completely. Lastly, proteins were suspended in 250 µl of Laemmli sample buffer comprised by 62.5 M Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, and 0.2 M dithiothreitol. After the protein quantification process, which was accomplished according to the micro Lowry modified method present in the literature [46], 15 µg of the protein solution were used to perform the Western Blot assay, using a Bolt 4 to 12% Bis-Tris, 1.0 mm, Mini Protein Gel (Thermo Fisher Scientific). After transferring the separated proteins to a PVDF membrane, using the Mini Blot Module kit (Life Technologies), the analysis was performed using a rabbit phospho-PKC (pan) antibody, with a loading control made out of a rabbit β-Actin antibody. Both the primary antibody and the horseradish peroxidase conjugated secondary one were diluted - the primary antibody at 1:1,000 and the anti-rabbit secondary at 1:10,000. Immobilion Western Chemiluminescent HRP Substrate (Merck) was used to get immunoreactivity; membrane images were captured using Thermo Fisher iBright FL1500 Imaging System; and the densitometric analysis was performed using the very iBright Analysis Software [47][48].

Results

Gene expression analysis

Gene expression was assessed after treatment with the previously listed inhibitors. As in the first stage, the control was the permissive medium condition. Graphs regarding the gene expressions data are shown in the figure below:

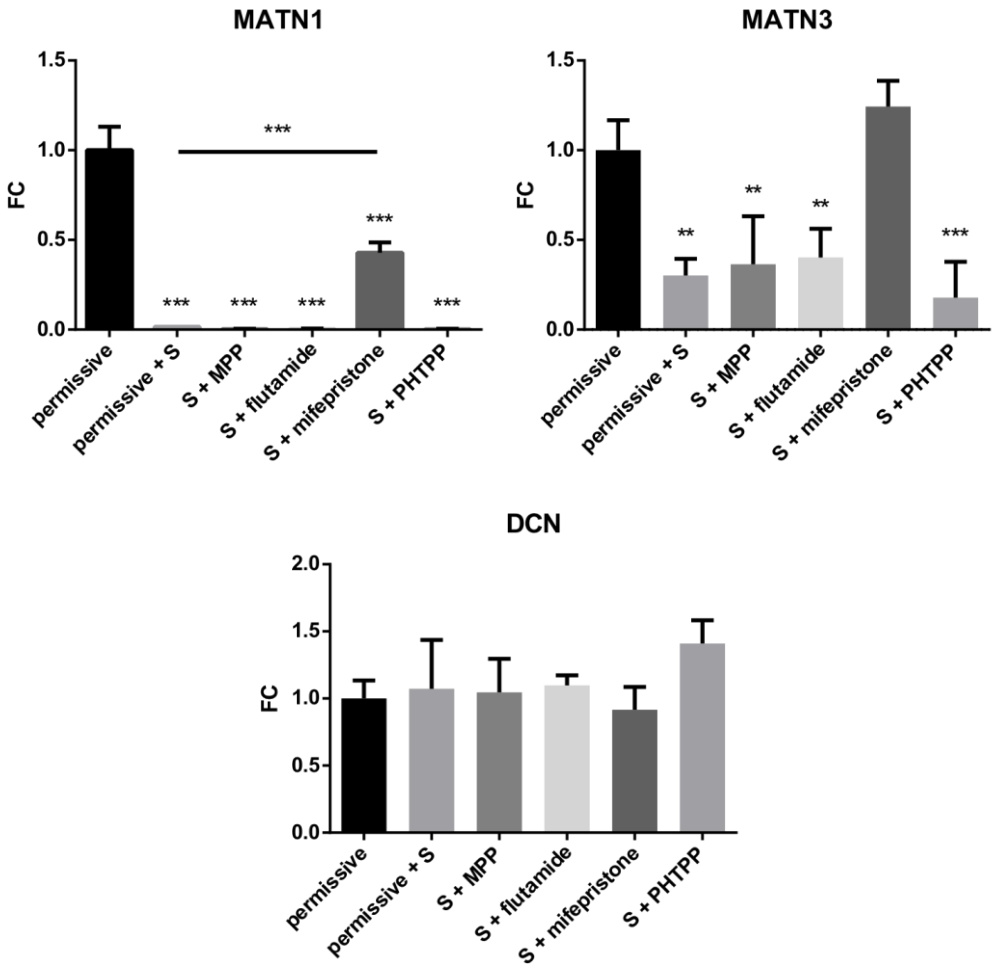


Figure 9. *These graphs show the fold change expression variations (FC) of MATN1, MATN3, and DCN genes for the various conditions used; gene expression was assessed with real-time PCR and analysed with Pfaffl formula [26]; p-value was considered significant for p-value < 0.05 (* p-value ≤ 0.05,** p-value ≤ 0.01, *** p-value ≤ 0.001). It was calculated with one-way ANOVA, followed by Dunnett's multiple comparisons test (comparison between permissive + S and S + mifepristone in MATN1 graphic was calculated via t test) performed with GraphPad 6.0. Media used were all permissive, besides the control one, all the others havean addition of 100 nM stanozolol, and the media indicated with the inhibitors' name each have the addition of the relative inhibitor in the following quantities: 10 nM MPP, 1 µM flutamide,20 nM mifepristone, and 1 µM PHTPP . All the expression variations were compared with the permissive medium indicated by an arrow.*

DCN expression ratio was assessed to make sure that the outcome was the same as the one produced in the first stage. As far asMATN1 and MATN3 expressions are concerned, the one condition characterized by the treatment with permissive medium supplemented with 100 nM stanozolol and 20 nM mifepristone was the one that most resembles the control gene expression and is the condition in which the inhibitor blocked the effect of the steroid. In S + mifepristone condition, MATN3 was expressed in the same way as the control

(fold change = 1.242674, SD = 0.143771, p-value = 0.3745), while MATN1 gene expression was significantly lower than the control (fold change = 0.4276778, SD = 0.057927, p-value < 0.0001). S + mifepristone MATN1 gene expression was, nevertheless, significantly higher than the permissive + S one (p-value = 0.0002).

Western blot assay

For each condition, we evaluated which phosphorylated PKC isoform was present and in what quantity. β-actin was used as loading control. The results are shown in the following figure.

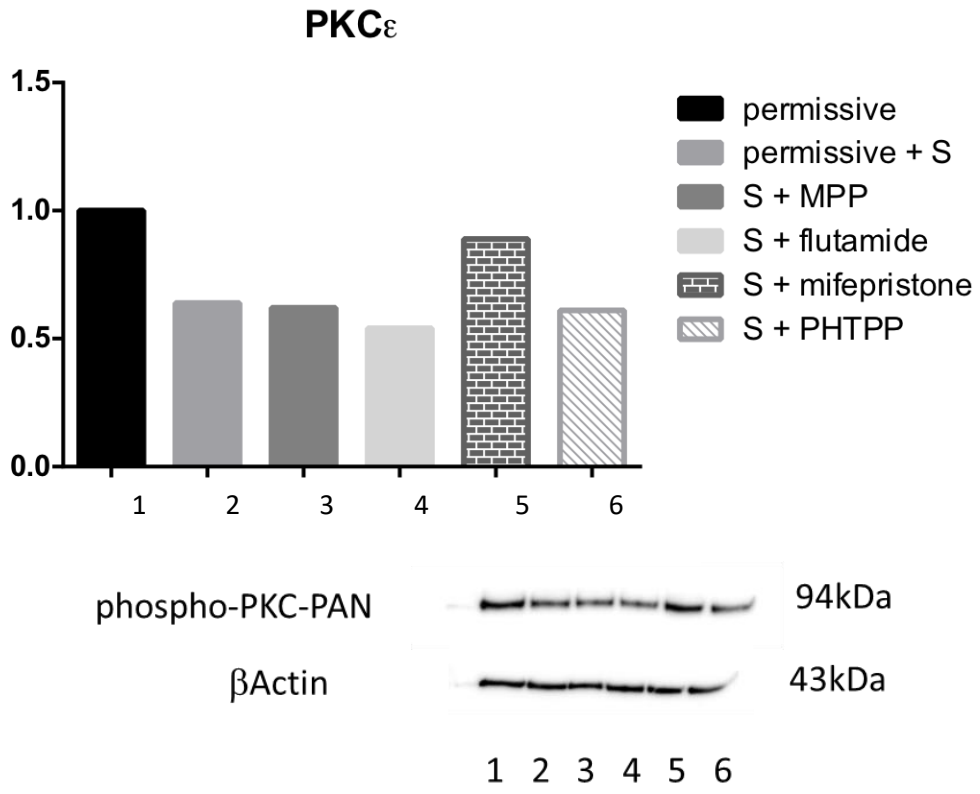


Figure 10. *This graph shows the outcome of the densitometric analysis performed with Thermo Fisher iBright Analysis Softwar;, the graph was created with GraphPad 6.0. Numbers indicate different conditions: 1 permissive medium, 2 permissive medium + 100 nM stanozolol, 3 permissive medium + 100 nM stanozolol + 10 nM MPP, 4 permissive medium + 100 nM stanozolol + 1 µM flutamide, 5 permissive medium + 100 nM stanozlol + 20 nM mifepristone, 6 permissive medium + 100 nMstanozolol + 1 µM PHTPP. The lower part of the figure is the image captured with Thermo Fisher iBright FL1500 Imaging System; the numbers indicate the same conditions mentioned above for the graph.*

According to the molecular weight (94 kDa), the PKC isoform detected was PKCε [49]. Consistent with real-time PCR results, western blot conditions' comparisons show a data trend that highlights, once again, that the inhibitor that produces less or completely non-evident stanozolol action is mifepristone.

Discussion

In the second stage, the gene expressions data of MATN1 and MATN3 highlighted how mifepristone treatment inhibits stanozolol's effect, thus causing the gene expression of these genes to resemble the control condition. This data indicates that, despite stanozolol analogy with DHT, it interacts with PR, in accordance with a thesis already present in the literature [30].

The data related to DCN expression indicates an unmarked differentiation efficiency in the observed conditions. The protein encoded by DCN gene is a cartilage ECM component that in micromass MSCs culture with dexamethasone treatment (absent in this study), is expressed early, in a timespan that goes from the second to the sixth day of culture [27].

Considering the induction of BMP2 expression via CD44 stimulation and the stanozolol related PGE_2 production [30], we can make a hypothesis about in which way and through which pathway stanozolol acts. Stanozolol appears to strengthen the differentiation stimuli due to BMP2 through PGE_2 action. Data regarding how BMP2 and PGE_2 interact in order to induce osteoid and cartilage differentiation are already present in literature. These data suggest that the two factors interact with each other and affect differentiation through a pathway that putatively involves MAPK (it also describes how PGE_2, like CD44, can induce BMP2). This phenomenon, as far as PGE_2 receptor interaction is concerned, features the involvement of different receptors according to the considered cell lineage. The receptor involved being EP2 in the bone cell lineage and EP4 in the cartilage cell lineage [50]; the latter, seemingly along with EP1 [51], is also the main receptor in MSCs' case [51]. PGE_2 induction, as stated, not only would strengthen MAPK induction, but also would induce BMP2, supporting the already present induction brought about by CD44 stimulation. A likely MAPK involvement is also suggested by the fact that its inhibition seems to lead to a partial inhibition of the

stanozolol effect [31]. PGE_2 seems to be capable of inducing chondrogenesis and inhibiting endochondral ossification through PKC and PKA action [53]. PGE_2 effect is different in different environments, even when considering the same cell type: for example, PGE_2 stimulate PKC in chondrocytes if they dwell in the growth zone, while it suppresses PKC if chondrocytes stay in the resting zone [54].

PKC seems to be involved, to some degree, in mesenchymal differentiation pathway, not only because it appears to mediate PGE_2 activity, but also because it seems to be involved in other important mesenchymal differentiation mechanisms, such as the one involving Ca^{2+} uptake [53][55][56][57].

PKCε activation levels (which was still phosphorylated at 12 days of treatment) not only confirm what was seen about mifepristone inhibition of stanozolol effect, but also constitute a data consistent with the pathway hypothesized for stanozolol. PKCε (along with PKCβ) appear to be of primary importance in mouse vertebrae formation, and it was also detected during embryonic development in chondrocytes derived from chicken tibiae. Furthermore, PKCε is overexpressed in the cartilage intermediate columnar layer in cases of osteoarthritic disease [55].

Although is now clear that stanozolol interacts with PR in cells from mesenchymal origin, its pathway of action remains unclear, especially regarding PGE_2 involvement, which seems to play a major role in this steroid's specific effect.

Chapter III

Appendix

The effect of COX2 inhibition in stanozolol induced differentiation in mesenchymal stem cell model

Abstract

Aim: The purpose of this study is to assess the role of PGE_2 in the stanozolol induced differentiation pathway. **Materials and methods:** hTERT MSC was the cell line used in this experiment. This experiment, as was true for the previous one, lasted for 12 days and was performed with micromass cell culture method. The experimental conditions were all differentiation permissive medium conditions. While positive control included the addition of 100 nM stanozolol, the other conditions included the addition of 2 μM indomethacin, 2 μM indomethacin and 100 nM stanozolol, and 20 nM mifepristone plus 100 nM stanzolol. The effects of the treatments were assessed through gene expression evaluation, obtained with real-time PCR assay. The genes of interest were: COX2, COL2A1, BMP2, SOX9, RUNX2, HES1, PPARγ, and MATN1. Statistical evaluation was achieved via one-way ANOVA, followed by Dunnett's multiple comparisons test, considering the results significant for p-value < 0.05. **Results:** COX2, responsible for PGE_2 production, was downregulated in the conditions with indomethacin and with mifepristone; this downregulation effect seemed to also be responsible for the expressions of BMP2, SOX9, RUNX2, and HES1. MATN1 expression was the same among indomethacin and mifepristone plus stanozolol conditions, and it was the same among stanozolol and stanozolol with indomethacin conditions, as well. **Discussion:** The results showed that mifepristone effectively inhibits stanozolol action through inhibition of PR activity and PGE_2 biosynthesis. PGE_2 induction appears to be of paramount importance in stanozolol induced differentiation.

Introduction

The data obtained in the previous experiment, although they clarify some details about stanozolol pathway of action, leave a few points unclear. This study aims to provide a better understanding about the role and importance of PGE_2 action in stanozolol treatment, thus shedding light on the cellular mechanisms induced by this steroid.

It was observed, throughout the whole manuscript, how stanozolol augments its regenerative potential when accompanied by a CD44 co-stimulation. CD44, when stimulated, is involved in both bone and cartilage regeneration pathways [14][16][17][18]. The literature and the results of the previous experiments show that CD44 induces BMP2 [14]. Furthermore, it was shown that stanozolol performs its function binding PR, but there is also evidence that its action is due to the binding of another receptor, which, though its nature remains unclear, is responsible for PGE_2 production [30].

PGE_2 action seems to be an important mediator in stanozolol treatment. Moreover, PGE_2 pathway interacts, through MAPK action, with the BMP2 induced differentiation, both in osteogenesis and in chondrogenesis. PGE_2 and BMP_2 pathways not only interact with each other, but they mutually induce each other. PGE_2 is, in fact, capable of inducing BMP2 production in MSCs, through EP4 receptor [51], and BMP2 seems to induce COX2 activity, causing PGE_2 biosynthesis [50]. Therefore, stanozolol treatment and CD44 stimulation could function as triggers capable of inducing these two intertwining pathways.

PGE_2 moderate concentrations seems to have an effect on the PKC induction [57] that, in turn, carries out an important role in MSCs' choice about which differentiation pathway to undertake [56]. Moreover, not only is stanozolol able to direct mesenchymal differentiation toward the formation of the characteristic anlagen interzone cells, but the PKCε isoform was found to be less phosphorylated at 12 days of culture as a consequence of stanozolol action (despite the fact that phosphorylation is not the only means to activating this specific protein). PKC, as well as BMP2 and PGE_2, mediates differentiation via MAPK pathway [55].

PGE_2 effect on PKC depends on the specific environment in which cells find themselves: PGE_2 induces PKC in growth zone chondrocytes, while it suppresses the factor in resting zone chondrocytes [54].

The objective of this study is to assess the role of PGE_2 in stanozolol treatment. COX2 inhibition was obtained with indomethacin. This molecule is a non-steroidal anti-inflammatory drug usually employed to inhibit COX1 and COX2 enzymes. If stanozolol acts through both PR binding and PGE_2 biosynthesis, its effect has to be partially blocked with COX2 inhibition due to indomethacin treatment. Furthermore, if BMP2 is induced by PGE_2 action, even if CD44 is stimulated by culture conditions, it has to be inhibited by indomethacin presence.

Materials and methods

Cell culture

As was the case before, hTERT MSC was the cell line used in this experiment. Micromass cell cultures were made out of 250,000 cells/micromass; after a few days' amplification in a 75 cm^2 flask in a DMEM/F12 medium with 10% FBS, 2 mM glutamine, and 1X penicillin/streptomycin, cells were detached with trypsin and were resuspendend in a minimal medium composed as follows: DMEM high glucose with 1% ITS+, 2 mM glutamine, and 1X penicillin/streptomycin. After a centrifugation at 500 g for 5 minutes, cell pellets were laid to rest for 48 h until the completion of micromass formation. Micromass cell cultures were made, as before, in airtight 5 ml conical tubes (once again, it is useful to remember that this situation makes the composition of the outer culture air irrelevant). After their formation, the minimal medium was changed with the experimental conditions: the control medium was a differentiation promoting DMEM high glucose medium with 2mM glutamine, 1% ITS+, 40 µg/ml proline, 200 µM ascorbate, 100 µg/ml sodium pyruvate, 10 ng/ml TGFβ3, and 1X penicillin/streptomycin; another condition was the same as the control one with the addition of 2 µM indomethacin; and the positive control medium was the same as the first cited control medium with the addition of 100 nM stanozolol. Lastly, the remaining two media used were constituted in the same way as the stanozolol positive control with the addition of a single inhibitor: there was 20 nM mifepristone in the PR inhibition medium, and, the last medium condition, intended to inhibit COX2 activity but in the presence of 100 nM stanozolol, was made with the addition to the mix of 2 µM indomethacin. The

experiment lasted 12 days, during which time cells were kept in a cell incubator at 37° C; media were changed every 2 or 3 days.

Gene expression analysis

RNA extraction was performed with Monarch Total RNA Miniprep Kit. First, each micromass was put into a 1.5 ml Eppendorf tube filled with 300 µl nuclease-free H_2O with the addition of 1% 2-mercaptoethanol, where it was homogenized with CAT X120 homogenizer. Afterwards, an equal volume of the kit DNA/RNA Protection Reagent was added, and, after that, another mix composed of 60 µl of kit Prot K Reaction Buffer plus 30 µl of kit's resuspended proteinase K was added. After a swift vortex step, protein digestion was performed, leaving the solution for 5 to 10 minutes at 55° C; samples were then vortexed and subsequently centrifuged at 16,000 g for 2 minutes. After the pellet was discarded, samples' volumes were doubled with the addition of an equal amount of kit RNA Lysis Buffer. These solutions were vortexed, and then the extraction protocol was followed according to "part 2: RNA binding and elution" manufacturer instructions. Lastly, the obtained RNA was resuspended in 50 µl of RNase-free H_2O.

RNA transcription into cDNA and real-time PCR were all performed according to the methods previously mentioned in chapter 1.

Primers used in this work are listed in the table below:

Gene name	Forward primer	Reverse primer
RPL15	GCAGCCATCAGGTAAGCCAAG	AGCGGACCCTCAGAAGAAAGC
COL2A1	GCAGCAAGAGCAAGGAGAAG	GTGGACAGCAGGCGTAGG
COX2	GGCTTCCATTGACCAGAGCAG	GCCGAGGCTTTTCTACCAGA
MATN1	CAGAGCACTACTTCTACAC	GAATTTCACCAGGGACTC
SOX9	GAGAGCGAGGAGGACAAGTTC	TCGTTCAGAAGTCTCCAGAGC
RUNX2	CCAGGCAGGCACAGTCTTC	GTCAGAGGTGGCAGTGTCATC
PPARγ	ACCACTCCCACTCCTTTGA	GCAGGCTCCACTTTGATTG
HES1	AGCACAGAAAGTCATCAAAGC	TTCCAGAATGTCCGCCTTC
BMP2	GTATCGCAGGCACTCAGGTC	GGTTGTTTTCCCACTCGTTTC

Table 6. *Table indicating primers that were used to perform real-time PCR assay*

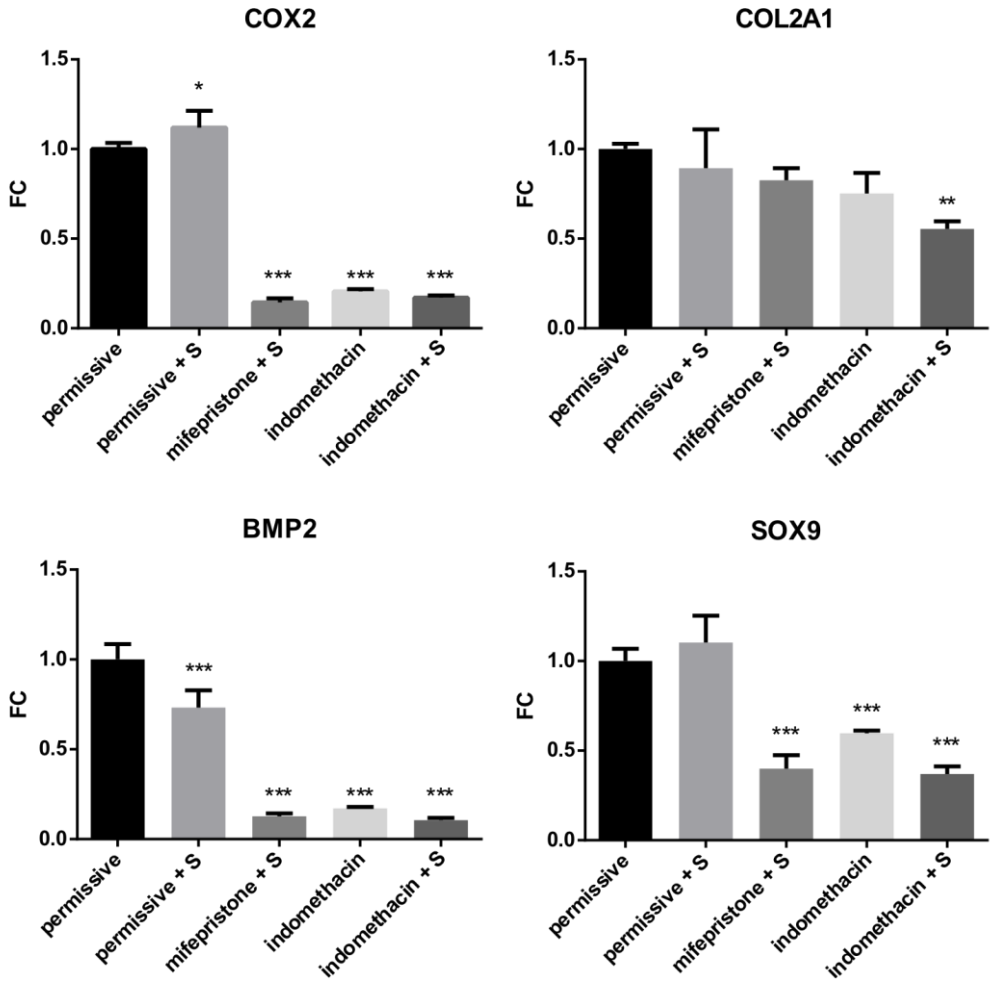
Statistical analysis

As was true in previous descriptions, PCR relative quantification was performed using the Pfaffl formula to assess the variation of the gene expression ratio [26]. P-value was assessed via one-way ANOVA, followed by Dunnett's multiple comparisons test, defining statistical significance at p-value < 0.05, with GraphPad Prism 6 software.

Results

Gene expression analysis

RPL15 was used as the housekeeping gene. The graphs obtained from the real-time PCR data are shown in the figure below. The control is the permissive medium gene expression.



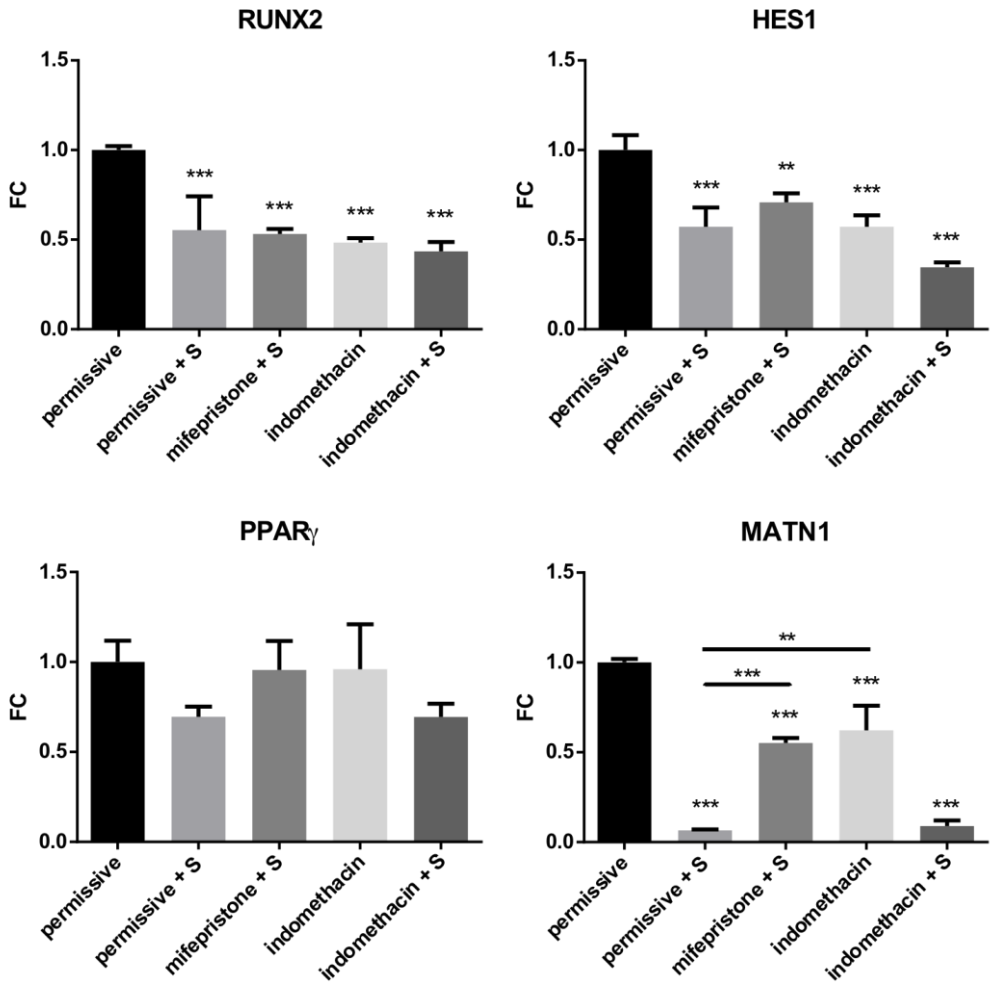


Figure 11. *These graphs show the fold change expression variations (FC) of COX2, COL2A1, BMP2, SOX9, RUNX2, HES1, PPARγ, and MATN1 genes for the various conditions used; gene expression was assessed with real-time PCR and analysed with Pfaffl formula [26]; p-value was considered significant for p-value < 0.05 (* p-value ≤ 0.05,** p-value ≤ 0.01, *** p-value ≤ 0.001). It was calculated with one-way ANOVA, followed by Dunnett's multiple comparisons test;MATN1 permissve+S vs mifepristone+S and permissive+S vs indomethacin were done via t test. All tests were performed with GraphPad 6.0.. All the expression variations were compared with the permissive medium.*

COX2 was significantly underexpressed in the mifepristone+S, indomethacin and indomethacin+S conditions (mifepristone+S fold change = 0.1439691, SD = 0.022625, p-value < 0.0001; indomethacin fold change = 0.2058232, SD = 0.012664, p-value < 0.0001; indomethacin+S fold change = 0.1703112, SD = 0.013089, p-value < 0.001). Conditions underexpressing COX2 have also significant BMP2 lower expression when compared to the control, underlining the aforementioned mutual interaction between PGE_2 and BMP2 pathways (mifespristone+S fold change = 0.1272702, SD = 0.015946, p-value < 0.0001;

indomethacin fold change = 0.171176, SD = 0.008594; p-value < 0.0001; indomethacin+S fold change = 0.1057461, SD = 0.012363, p-value < 0.0001). The only exception was permissive+S condition (COX2 fold change = 1.119915, SD = 0.093546, p-value = 0.0319), where BMP2 expression was still slightly lower compared to the control, even though significant (fold change = 0.7312612, SD = 0.096769; p-value = 0.0008). SOX9, as well as BMP2, is underexpressed in the conditions containing mifepristone and indomethacin with or without stanozolol (mifepristone+S fold change = 0.3998231, SD = 0.074523, p-value < 0.0001; indomethacin fold change = 0.5973534, SD = 0.014587, p-value = 0.0005; indomethacin+S fold change = 0.369070, SD = 0.043180, p-value < 0.0001). RUNX2 exhibits the same basic trend as HES1. MATN1 showed a significant underexpression in all of the conditions tested compared to the control. Furthermore, there were no differences between the mifepristone+S and indomethacin MATN1 gene expressions (p-value = 0.4390), while the comparison between permissive+S and mifepristone+S (p-value < 0.0001) and permissive+S and indomethacin (p-value = 0.0022) showed significant gene expression variations. Regarding MATN1, there was not any difference between permissive+S and indomethacin+S conditions (p-value = 0.2620).

Discussion

The genes whose expression was analysed and that were not cited earlier are as follows:

COX2: Cyclooxygenase 2 - it is an inducible enzyme, and it is responsible for prostaglandin production (such as PGE_2), which is an inflammation mediator; it is also involved in mitosis induction.

HES1: HES1 is involved in cell differentiation. It appears to be responsible for the mesoderm formation during embryogenesis, and also in MSCs chondrogenic commitment [58], and in bone development [59][60]. It is activated by RBPjκ action induced by NICD, the NOTCH receptor cleaved intracellular domain.

PPARγ: It is the master switch of adipogenesis. When present in the bone formation process, it binds RUNX2, preventing it from functioning as a transcription factor [61].

Functional COX2 inhibition, due to the presence of indomethacin, downregulates the enzyme's mRNA synthesis; the same situation was also seen in a preliminary experiment with the use of 1 μM hydrocortisone (data not shown in this manuscript). What was unexpected was that mifepristone seems to act as a COX2 inhibitor, as well. Mifepristone, which is a glucocorticoid receptor (GR) antagonist, can act as its agonist when GR concentration rises. This specific situation is not due to molecule $EC_{50,}$ since this characteristic is independent from receptor levels [62]. Furthermore, it was also shown that mifepristone can inhibit both PGE_2 biosynthesis and arachidonic acid release with two distinct pathways, through an action that mimics glucocorticoid effect [63]. Regarding permissive medium's COX2 expression, MSCs naturally produce IL-1 [64], which is known to induce COX2 [65].

As mentioned in the last experiment's discussion, BMP2 and PGE_2 pathways are two intertwining pathways that augment and induce each other mutually [50]. BMP2 is involved in chondrogenesis, and that is, in fact, attested by the detected SOX9 expression pattern.

RUNX2 and HES1 follow the same trend, as well. Regarding the permissive and permissive+S conditions, the stanozolol presence induces MSCs to differentiate into cells that will be part of the anlagen interzone, while its absence put the cells through a differentiation pathway that will ultimately lead to endochondral ossification. Following the logic of this statement, it is obvious that these factors are upregulated in the condition characterized by stanozolol absence. Apart from, RUNX2, which is the osteogenesis master switch, not only is HES1 an important factor in bone development during embryogenesis (as well as in bone fracture healing) [58][60], but its overexpression manages to suppress chondrogenesis in micromass cell culture, and its suppression improves chondrogenesis successfully, as well as causing an overexpression of SOX9 and COL2A1 genes [59].

All considered, mifepristone is the perfect stanozolol inhibitor since it inhibits both PR activity and PGE_2 biosynthesis. From the results, it appears that the PGE_2 pathway is the main pathway involved in stanozolol differentiation induction, but it is not the only one. The observed MATN1 downregulation can be explained only with the inference that stanozolol binds PR, as well. Upon observation of the MATN1 graph, it becomes clear that mifepristone's stanozolol inhibition makes MATN1 expression the same as the indomethacin treatment, which is capable of blocking PGE_2 biosynthesis. Furthermore, MATN1 downregulation is due to

the PR-stanozolol interaction only, as the gene underexpression appears the same when the conditions with stanozolol treatment and stanozolol co-treatment with indomethacin are compared.

Conclusion

The experiments described in this manuscript have shown stanozolol's ability to induce osteochondral regeneration. Stanozolol enhances both chondrogenesis and osteogenesis and augments its effect when accompanied by a CD44 stimulation. It was described how, in chondrocyte micromass culture, a culture condition intended to simulate adult cartilage environment, and in a preosteocytic model along with a HA hydrogel co-treatment, stanozolol performed its action better, improving its differentiation effect. In the end, when it comes to the treatment of MSCs micromass cultures with the steroid, the task of figuring out which pathway it induces in order to perform its function was achieved.

In the literature, there are different explanations intended to clarify which receptor stanozolol interacts with and which response it induces. The answers obtained with the performed experiments point towards the explanation given by *Ellis et al.* in their work, which illustrated that stanozolol interacts both with PR and an unknown receptor not present in every cell type [30]. In *Zhu et al.,* stanozolol is described as an ERα ligand, and it is explained that it performs its function through this receptor [31]. Nevertheless, stanozolol can induce ERα activation even without the classical ligand-receptor interaction. As seen during the experiments, the hypothesized interaction between the PGE_2 pathway, induced by stanozolol, and the BMP2 pathway, induced by CD44 stimulation, create some sort of "bottleneck" toward differentiation induction, constituted by MAPK activation [50]. MAPK can lead to a ERα activation, which differs from the ligand-receptor interaction relatively to the phosphorylated serine residues: While with the classical ligand-receptor interaction, receptor's serine 118 would be phosphorylated, in the case of the MAPK activation, the receptor would be phosphorylated in two different loci, serine 118, as before, and serine 167 [31]. The classic ERα inhibitors, like MPP or ICI 182.780, prevent the phosphorylation only of the serine 118 [67]. The authors of the aforementioned *Zhu et al.* manuscript noticed this occurrence and concluded that stanozolol putatively activates ERα both ways: from direct interaction and with the MAPK involvement. Furthermore, they do not specify if the DMEM/F12 they used as a medium ingredient contained phenol red as pH indicator. This factor is important since this molecule can mimic estradiol [68] and, in this way, could lead to ERα direct stimulation. Moreover, it deserves to be mentioned that they did not test stanozolol interaction with any other receptors other than ERα and AR.

According to what is suggested by the findings of the experiments detailed in this manuscript, it can be hypothesized that, since stanozolol's effect seems to be mainly mediated by PGE_2 biosynthesis obtained through the steroid interaction with an unknown receptor, stanozolol does not have a tissue specific effect per se [29], but it can perform its effect, particularly in those tissues whose cells express this receptor, as is the case with skin fibroblasts, but not so, for example, in the case of synovial fibroblasts [30].

All in all, relying on literature and on the results from the experiments, stanozolol's pathway of action, whose efficiency is augmented in presence of CD44 stimulation, can be concretely hypothesized: In MSC micromass culture, stanozolol interacts both with PR receptor and with an unknown receptor. This latter interaction induces PGE_2 biosynthesis which enhances BMP2 induction (and, in turn, is enhanced by BMP2), which is already present due to CD44 stimulation. These two pathways induce osteochondral differentiation through MAPK activation [50]. The stanozolol-PR interaction is responsible, putatively through PKCε underactivation, of the suppression of MATN1 gene expression, making MSCs differentiate into cells that, during embryogenesis, constitute anlagen interzones from which joint chondrocytes will form.

There has to be some kind of interaction between PGE_2 stimulus and the MATN1 induction due to PKCε, since we observed a significant repression of MATN1 gene expression following COX2 inhibition.

The pathway, briefly summarized, is rendered graphically in the figure below (created with BioRender.com):

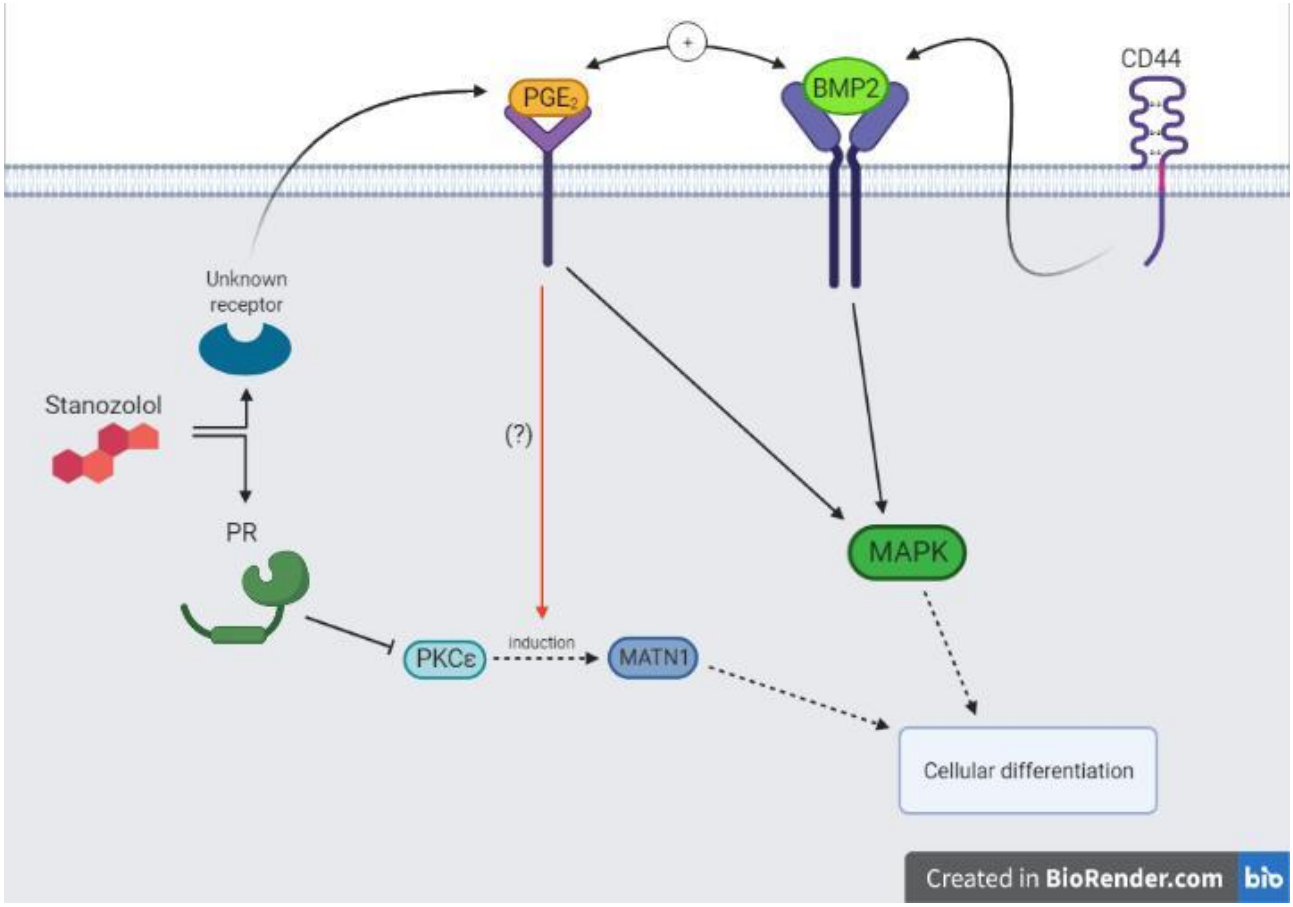


Figure 12. *This image represents the hypothesized stanozolol pathway in MSC micromass culture. Stanozolol interacts with two receptors: it interacts with PR which is responsible for MATN1 gene expression's downregulation, putatively through PKCε inactivation, and it interacts with an unknown receptor that is responsible for PGE_2 biosynthesis. Due to culture method, BMP2 is already induced by CD44 action, and both PGE_2 and BMP2 enhance the biosynthesis and effect each other mutually. These two intertwining pathways induce osteochondral differentiation through MAPK activity [50]. (image created with BioRender.com)*

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