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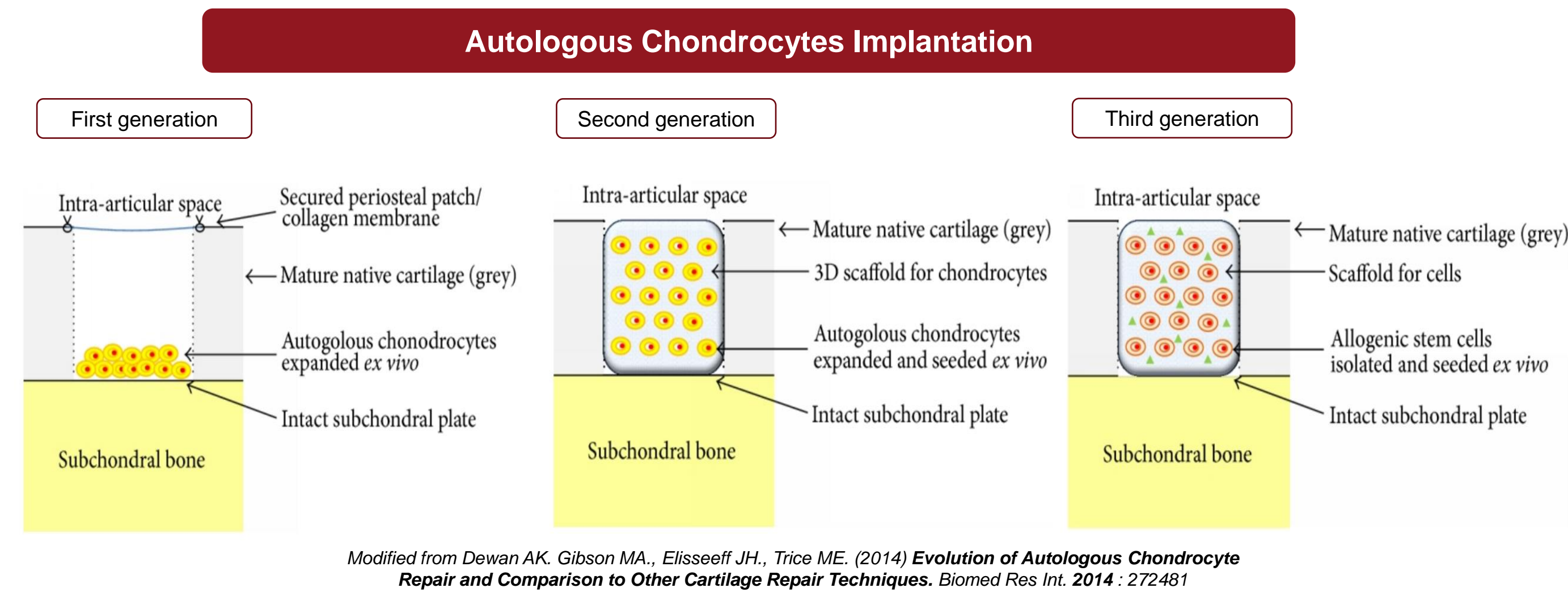
Improvement of the chondrocyte-specific phenotype upon equine bone marrow mesenchymal stem cell differentiation. Influence of TGF- β 1 or TGF- β 3, associated with BMP-2 and type I collagen siRNAs

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Introduction

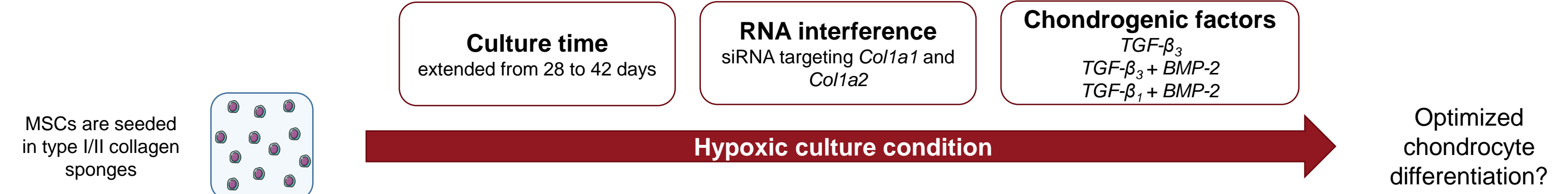
Articular cartilage is a tissue characterized by its poor intrinsic capacity for self-repair. This tissue is frequently altered upon trauma or in osteoarthritis (OA), a degenerative disease that is currently incurable. Consequently, cartilage markers, such as type II collagen, are degraded whereas atypic molecules, such as type I collagen, are newly synthesized. Another essential phenomenon occurring in OA is the upregulation of Htra1, a serine protease targeting upstream receptors of signalling pathways involved in the synthesis of articular cartilage markers. OA incurs considerable economic loss for the equine sector. In the view to develop new therapies for humans and horses, significant progress in tissue engineering has led to the emergence of new generations of cartilage therapy. Matrix-associated autologous chondrocyte implantation is an advanced 3D cell-based therapy that holds promise for cartilage repair. The aim of this study is to improve the autologous chondrocyte implantation strategy by enhancing the chondrogenic differentiation of mesenchymal stem cells (MSCs) in order to increase the type II collagen/ type I collagen ratio.



Methods

This study aims to improve the autologous chondrocyte implantation technique by using equine MSCs from bone marrow differentiated into chondrocytes that can be implanted in the chondral lesion. Our previous chondrogenic differentiation protocol lasted 14 days and consisted in an hypoxic 3D culture of MSCs in the presence of chondrogenic factors (BMP-2, TGF- β 1) and siRNAs targeting only the alpha 1 chain of type I collagen and Htra1.

Here, we explored three parameters that could influence MSC differentiation: culture time, chondrogenic factors and siRNA targeting the alpha 2 chain of the type I collagen since type I collagen is a heterodimer composed of two alpha 1 and one alpha 2 chain.

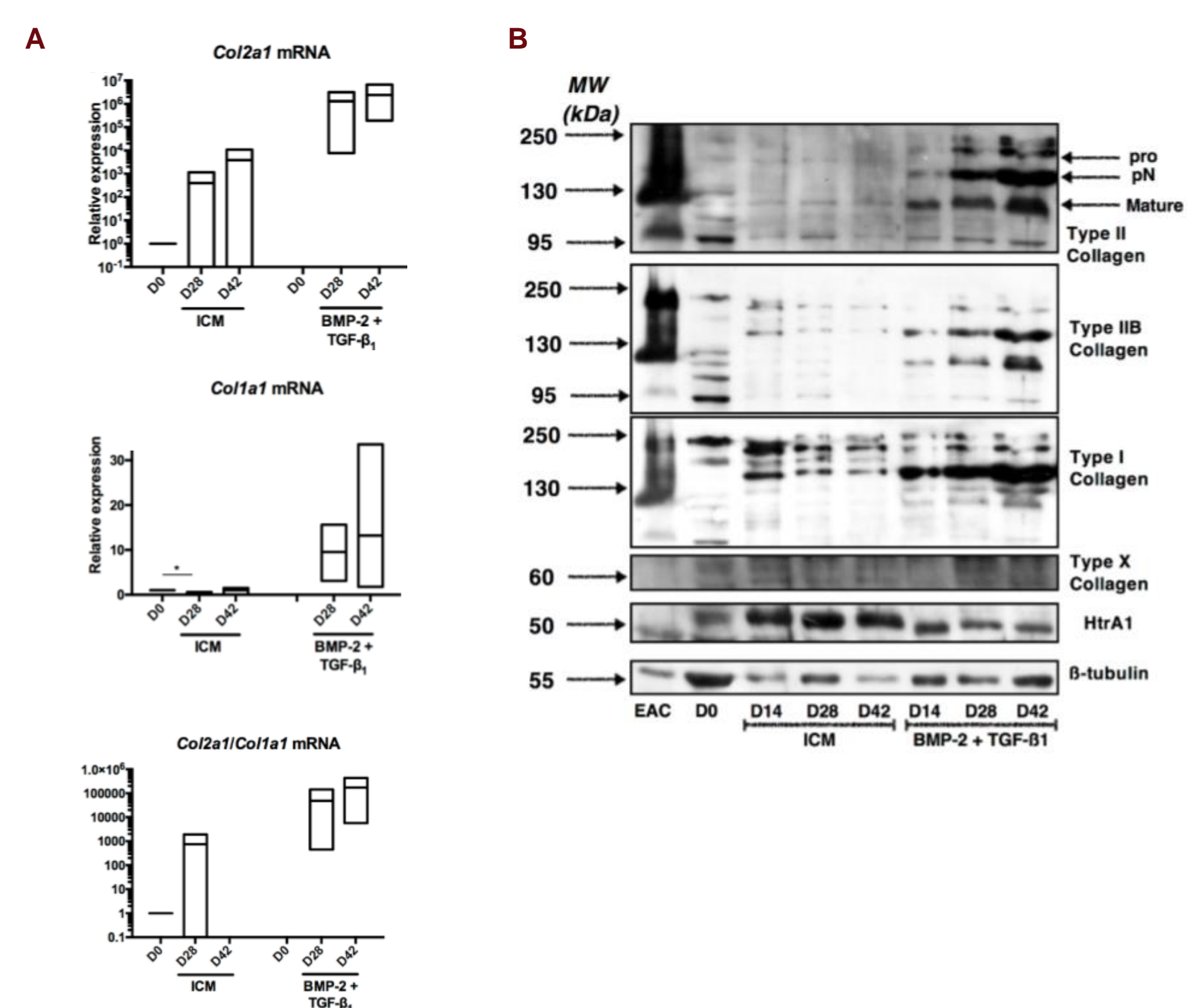


Results

1- Influence of culture time

Increase in the culture time of MSCs in the presence of BMP-2 and TGF- β 1 until 42 days and its influence on the expression of hyaline articular cartilage non specific and specific markers at the mRNA level (A) and protein level (B).

Equine MSCs derived from bone marrow were amplified and seeded in collagen sponges at P4 (n=3). They were then cultured in hypoxia (3% O₂) for 14, 28 and 42 days in the presence of incomplete chondrogenic medium (ICM) alone or supplemented with BMP-2 (50 ng/ml) and TGF- β 1 (10 ng/ml) (BMP-2 + TGF- β 1). D0: stem cells cultured in amplification medium at P4; EAC: equine articular chondrocytes.

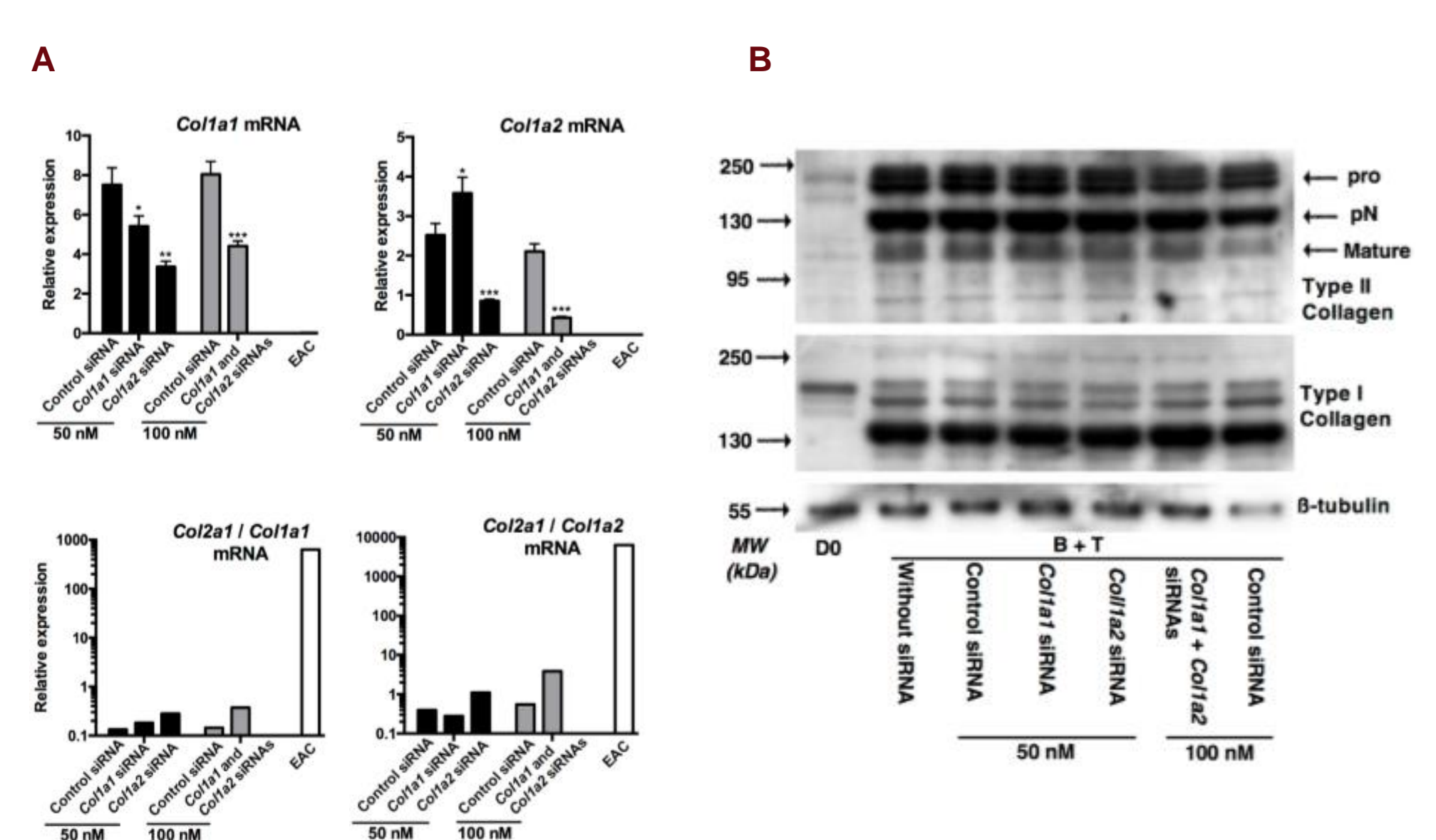


- > Culture time extension leads to an increase of the expression of chondrocyte markers, but also of type I collagen.
- > The association of BMP-2 and TGF- β 1 decreases Htra1 protein expression.
- > The *Col2a1/Col1a1* ratio at the mRNA level is not modulated from D28 to D42.

2- Effect of RNA interference

Effect of *Col1a1* and/or *Col1a2* siRNAs on the mRNA (A) and protein (B) levels of specific and nonspecific markers of chondrocytes derived from MSCs cultured 42 days.

Equine MSCs derived from BM were amplified and seeded in type I/II collagen sponges at P4 (n=3). They were then cultured in hypoxia (3% O₂) for 42 days in incomplete chondrogenic medium (ICM) in the presence of BMP-2 (50 ng/ml) and TGF- β 1 (10 ng/ml). The MSCs were transfected with a control siRNA or *Col1a1* and/or *Col1a2* siRNAs (50 nM of each siRNA was transfected). D0: stem cells cultured in amplification medium at P4; EAC: equine articular chondrocytes.

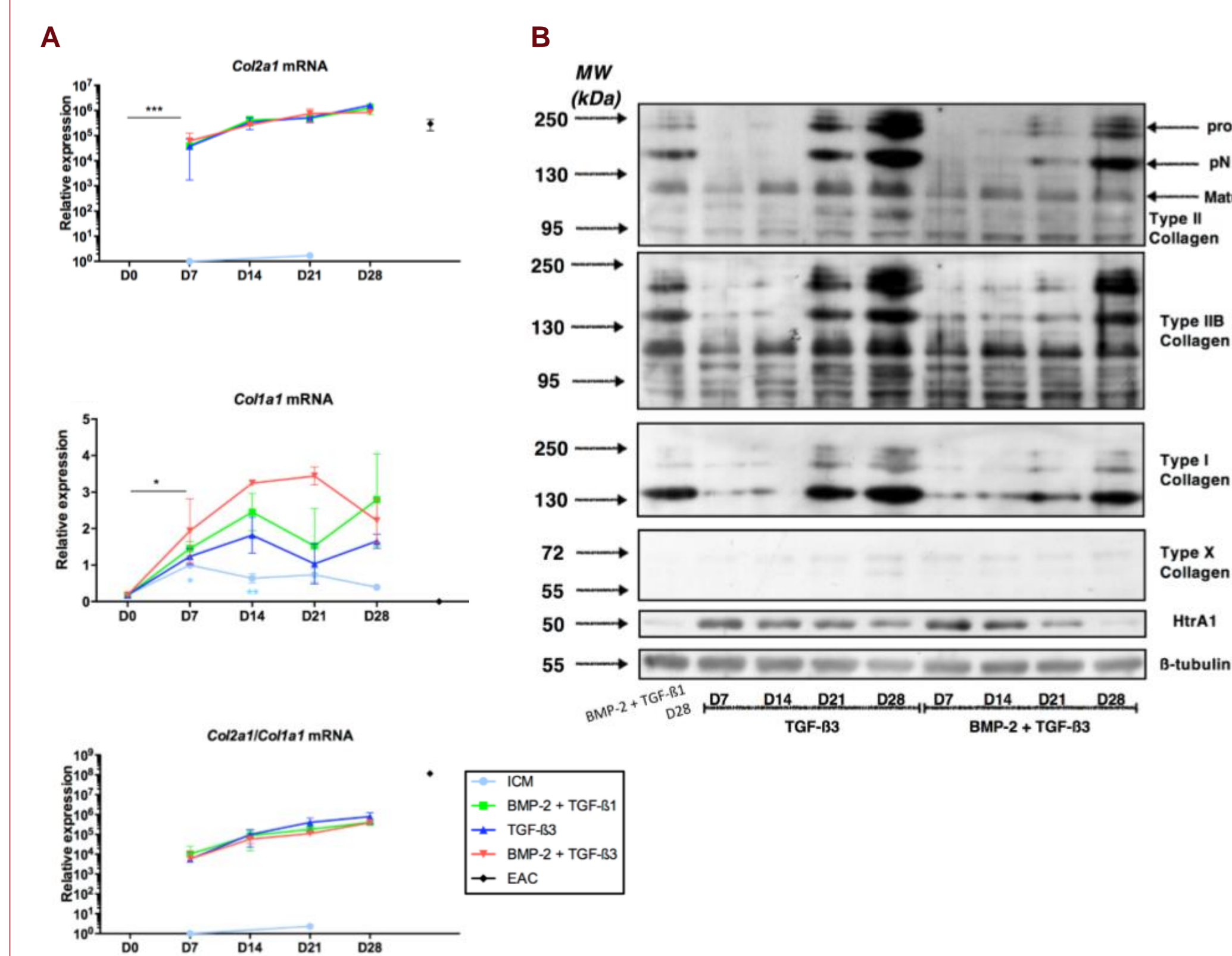


- > RNA interference targeting *Col1a2* leads to a more significant knockdown, at the mRNA level, compared to a conventional strategy targeting *Col1a1*. The combination of the two siRNA leads to the better *Col2a1/Col1a1* ratio.
- > Type I collagen protein expression is not significantly modulated whatever the siRNA used.

3-BMP-2+TGF- β 1 or BMP-2+TGF- β 3 for a better chondrogenic effect?

Expression of mRNAs (A) and proteins (B) of articular cartilage specific markers during chondrogenic differentiation of MSCs cultured in the presence of BMP-2 + TGF- β 1 or TGF- β 3 alone or in combination with BMP-2.

Equine MSCs derived from bone marrow were amplified and seeded in collagen sponges at P4 (n = 2). They were then grown in hypoxia (3% O₂) for 7, 14, 21 and 28 days (D7, D14, D21 and D28, respectively) in the presence of incomplete chondrogenic medium (ICM) alone or enriched with BMP-2 (50 ng/ml) and TGF- β 1 (10 ng/ml) (BMP-2 + TGF- β 1), TGF- β 3 (10 ng/ml) or BMP-2 (50 ng/ml) and TGF- β 3 (10 ng/ml) (BMP-2 + TGF- β 3). D0: stem cells cultured in amplification medium at P4; EAC: equine articular chondrocytes.



- > TGF- β 3 alone showed promising results at both mRNA and protein levels, but, regarding the articular cartilage atypic type I collagen, the previously tested association of BMP-2 and TGF- β 1 better limits the expression of type I collagen at the protein level. Moreover, the use of TGF- β 3 alone induces a higher expression of the Htra1 serine protease compared to the previously tested growth factors association.

Conclusion

These data indicate that the original BMP-2 + TGF- β 1 association seems to be a better solution for the chondrocyte differentiation of equine BM-MSCs, despite the higher expression of type II collagen when TGF- β 3 is used. The cartilage extracellular matrix (ECM) components also showed enhanced expression for longer culture times, up to 6 weeks. However, the increase in ECM production was moderate after 14 days of culture, and the best compromise between high ECM synthesis and minimal production of type I collagen still needs to be clarified. Thus, culture times between 14 and 28 days remain the best way to obtain chondrocytes like cells. Before considering *in vivo* trials in an equine clinical setting, the knockdown strategy should be improved. However, to date, our differentiation protocol may be applicable for pre-clinical trials in the horse to help to decide on the best *in vitro* culture conditions to produce a hyaline-type articular cartilage *in vivo*.

