RESEARCH HIGHLIGHT

The ephrin-B2/EphB4 system is required in musculoskeletal development and protects the articulation during osteoarthritis: a research highlight

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Ephrin ligands and their Eph receptors have been implicated in the control of extracellular matrix of some tissues. Although ephrin-B2 and its specific receptor EphB4 were found to be involved in postembryonic control of bone homeostasis, their roles were unclear in musculoskeletal growth and development as well as in osteoarthritis pathology. The role of this ephrin system in musculoskeletal growth and development was delineated *in vivo* using a cartilage-specific ephrin-B2 knockout mouse model. Its role in osteoarthritis *in vivo* was explored in mice using a bone-specific overexpression of EphB4 in which osteoarthritis was induced, and *in vitro* in human osteoarthritic subchondral bone osteoblasts and chondrocytes. *In vivo*, ephrin-B2 demonstrated to be essential for normal long bone growth and development and its absence in cartilage led to knee and hip osteoarthritis features in aged mice. *In vitro* data showed that the ephrin-B2-induced EphB4 receptor positively impacted the abnormal metabolism of both osteoarthritic subchondral bone osteoblasts and chondrocytes. The bone-specific EphB4 overexpression in mice validated the *in vitro* data in that it had beneficial effects not only on the osteoarthritic subchondral bone but also on the cartilage and synovial membrane, and further substantiated the hypothesis that by prophylactically protecting the subchondral bone, the genesis of osteoarthritis could be, at least in part, inhibited. In the context of identifying new candidates targeting osteoarthritis progression, this ephrin system is extremely attractive as a potential novel therapeutic avenue, as therapies having a more global articular approach may prove to be the most successful to arrest or slow the progression of this disease.

Keywords: ephrin; Eph receptor; bone development; osteoarthritis; cartilage; subchondral bone; synovial membrane

Introduction

Ephrin

The erythropoietin-producing hepatocyte kinases (Ephs) are receptors constituting the largest subfamily of membranous receptor tyrosine kinases, representing about 25% of this receptor family [1]. Originally identified as axon guidance molecules [2], they have since been shown in many organs and tissues and are known to control extracellular

matrix of some tissues with widespread roles in normal physiology and disease [3]. There are a total of 15 Ephs classified by sequence homology into subfamily A with 9 members, and B with 6 members. Not all are expressed in a given species; for example, humans and mice do not express EphB5 [2, 4]. Ephs bind to ephrin ligands, which are also cell surface molecules [1]. There are 9 ephrins divided into A and B subfamilies based on the manner in which they anchor to the cell surface. In general, EphA members preferentially interact with ephrins-A, and EphB with ephrins-B [2, 4].

A characteristic of this system is its ability to exhibit bidirectional signaling [5-8]. The Eph receptors initiate signal transduction through autophosphorylation after ligand binding; however, the ephrin ligands also have the ability to initiate receptor-like active signaling. Signaling through Eph receptors is considered forward and through ephrin ligands reverse. For example, in the musculoskeletal system, this is well illustrated in bone where several ephrin and Eph family members were shown to regulate bone development, maintenance, and repair [7, 9-11]. In bone, the osteoclasts only express ephrin-B with no detectable EphB receptors [7], while osteoblasts express both ephrin-B and EphB receptors [12]. Hence, ephrin-B1 and B2 on osteoclasts could forwardly trigger EphB4 on osteoblasts to stimulate their differentiation, while reverse signaling from EphB4 on osteoblasts to ephrin-B1 and B2 could repress osteoclast differentiation [7]. The overall outcome of such interaction favours bone formation. Similarly, EphA2 on osteoblasts could trigger reverse signalling via ephrin-A2 to promote osteoclast differentiation and inhibit osteoblast differentiation [10]. However, the initial concept that functional ephrin/Eph interactions occur only between the surfaces of opposing cells has been challenged as it has been shown that Ephs and ephrins are often co-expressed by the same cells. Moreover, this system was reported to not always function bidirectionally: ephrin can simply act as a ligand for the Eph without any requirement for ephrin signalling [13].

Osteoarthritis

Osteoarthritis is by far the most common musculoskeletal disease affecting millions of individuals worldwide, accounting for 40-60% of musculoskeletal system degeneration [14]. It represents a major cause of morbidity and disability, particularly in the second half of life. It is a chronic disease with a prevalence of about 12%, of which approximately 65% are 60 years of age and older. With the ageing of the world's population, the burden of this disease has been steadily gaining importance in the last few decades and it is expected to increase by about 40% by 2025 [14]. The disease develops progressively over decades and mainly affects the diarthrodial joints. It results in joint tissue degeneration that culminates in pain, loss of motion, and instability.

Although important advances in understanding the pathophysiological processes of this disease have been made, today's treatment still focuses mainly on improving the symptoms. However, despite treatment with conventional drugs, most individuals with osteoarthritis continue to experience pain. Preservation of joint tissue structure is the key to curing this disease. Therefore, the next generation of osteoarthritis drugs should target its cause, i.e. prevent the destruction of the joint tissues. The ultimate vision for treatment has been to find agents that can reduce or stop the disease progression. Thus far, no such treatment has been approved by regulatory agencies. Hence, identifying new therapeutic targets to stop or slow down the disease process is critical.

Osteoarthritis affects all the articular tissues. Although its hallmark is the progressive degeneration of the cartilage, subchondral bone alterations and synovial membrane remodeling and inflammation are now recognized as active components of the disease, which, with the cartilage, are considered to constitute an interdependent functional unit, demonstrating cross-talk between these tissues [15].

Objectives

Although EphB4 and its main ligand ephrin-B2 were found to be involved in the postembryonic control of bone homeostasis, their role in musculoskeletal growth and development as well as in osteoarthritis were addressed only recently. These two topics will be the focus of this highlight, as a better understanding of the mechanisms that regulate the development and growth of bone and cartilage may provide insight into the antecedents of joint diseases such as osteoarthritis.

Ephrin-B2 in musculoskeletal growth and development

Skeletal growth involves endochondral bone formation at the growth plate, which causes elongation of the metaphyses, intramembranous bone formation at the periosteal surface, widening of the diaphysis and chondrogenesis at the articular surface, producing an expansion of the epiphyses.

The majority of the skeleton arises by endochondral ossification, whereby cartilaginous templates expand and are resorbed by osteoclasts, then replaced by osteoblastic bone formation. To study the role of ephrin-B2 *in vivo* in musculoskeletal growth and development, a cartilage-specific ephrin-B2 knockout (KO) in mice [16] was employed. This approach was chosen for several reasons:

germ-line mutation of this ephrin in mice leads to embryonic lethality in homozygous nulls [17, 18]; both EphB4 and ephrin-B2 are expressed in the bone and in the chondrocytes of the growth plate [9, 19]; growth of articular cartilage (chondrocyte differentiation in juvenile mice) implicated ephrin receptor signaling pathways [20]; EphB4 enhances the process of endochondral ossification bone repair [21]; and the presence and activity of both ephrin-B2 and EphB4 have been demonstrated in adult cartilage (chondrocytes) (see below, [22]).

The genetically modified mice harboring a cartilage-specific deletion of ephrin-B2 were generated using the Cre-Lox methodology, in which mice carrying Cre recombinase under the control of the type II collagen promoter (the collagen specific for cartilage), Efnb2^{Col2}KO, were used to induce specific recombination in chondrocytes [16].

Data first showed that the cartilage-conditional Efnb2^{Col2}KO mice were smaller in size compared to control (Efnb2$^{fl/fl}$) mice and some regions of the limbs exhibited thinner skeletal staining on the mutant mice than those of the controls. They had smaller body weight and length, and significant reduction in bone length of the tibia, femur and humerus.

At postnatal day 0 (P0), the growth plate of Efnb2^{Col2}KO mice, although presenting no differences in the reserve or proliferating zones, showed a disorganized hypertrophic cartilage zone with a significantly increased staining of type X collagen and a decrease in mineralized cartilage matrix at the chondro-osseous junction. This suggests that, in these mutant mice, there is no abnormal growth or differentiation of the chondrocytes, but an abnormal chondrocyte metabolism.

At P15, the mutant mice displayed a delay at the secondary center of ossification, significantly decreased vascular endothelial growth factor (VEGF) levels, a disturbed mineralization associated with less bone volume and trabecular thickness, as well as a decrease in the recruitment and invasion of tartrate resistant acid phosphatase (TRAP) positive cells, suggesting that ephrin-B2 delayed vascular invasion into hypertrophic chondrocytes, regulating angiogenesis at the chondro-osseous junction, thus facilitating endochondral bone development.

Moreover, in the Efnb2^{Col2}KO mice, bone mineral density analysis revealed a significant reduction in the whole body, femoral head, and spine at P21 and 8 weeks old. At 1 year, although values were lower for each of the abovementioned bones studied, statistical significance was reached only for the femoral head, suggesting that the independent and highly coordinated bone remodeling process may have superseded the effect of the lack of ephrin-B2 in an attempt to take over the long bone development.

The micro-architecture of the femur and tibia evaluated in 8-week-old mice revealed a reduction in the mineralized tissue by about 35% as well as the trabecular thickness and trabecular separation. A significant reduction in the trabecular number was found only in the tibia. This decrease in mineralized tissue in the Efnb2^{Col2}KO mice could be a consequence of improper cartilage matrix degradation caused by ephrin deficiency in late hypertrophic chondrocytes.

Importantly, at 1 year, the mutant mice demonstrated osteoarthritic features of cartilage degeneration in both the knee and hip. This was seen radiographically, where the ephrin KO mice exhibited a collapse of the joint with a decreased joint space, as well as histologically.

Together, these data demonstrated that ephrin-B2 is essential for normal long bone growth and development and its absence leads to knee and hip osteoarthritis features in aged mice.

These data are supported by a recently published study [23] also investigating the role of ephrin-B2 in endochondral ossification but using the genetically modified mice carrying Cre recombinase under the control of the osterix-1 (Osx1) promoter, a zinc finger-containing transcription factor essential for osteoblast differentiation and bone formation, Osx1Cre.Efnb2KO. Such ephrin-B2 gene recombination targets not only the osteoblast lineage but also pre-hypertrophic and hypertrophic chondrocytes [24, 25].

Ephrin-B2/Ephb4 in osteoarthritis

***In vitro*: human osteoarthritic subchondral bone osteoblasts**

To further explore the role of this system in osteoarthritis, the possible implication of EphB4 receptor activation by ephrin-B2 was examined first *in vitro* in human osteoarthritic subchondral bone osteoblasts [12]. These cells were used as EphB4 is produced by osteoblasts and osteocytes, but not by osteoclasts [7]. Moreover, data showed that an abnormal process occurs in the subchondral bone osteoblasts during osteoarthritis [26].

Data revealed [12] that both ephrin-B2 and EphB4 are expressed on and produced by human subchondral bone osteoblasts and that the functional consequence of the ephrin-B2-activated EphB4 positively affects abnormal

metabolism by reducing the remodeling process of this tissue and associated bone remodeling factors. The latter includes the inflammatory factors interleukin (IL)-1β and IL-6, and the metalloproteinases (MMP) MMP-1, MMP-9, and MMP-13. Moreover, the receptor activator of nuclear factor κB ligand (RANKL), a factor synthesized by cells of osteoblast lineage and essential for mediating bone resorption through the enhancement of osteoclast differentiation and proliferation, was also significantly inhibited. Interestingly, another important member of this family, osteoprotegerin (OPG), also secreted by osteoblasts and acting as a decoy receptor for RANKL, was not regulated. This in turn disturbed the equilibrium between these two factors with the net outcome of an increased ratio of OPG to RANKL, and therefore a reduction in the osteoclastogenesis process. The effect of EphB4 activation on these abnormal cells appears to occur through a down-regulation of the phosphatidylinositol 3-kinase (PI 3-kinase)/Akt signaling pathway.

This study was the first to provide evidence that EphB4 activation by ephrin-B2 in human osteoarthritic subchondral bone could positively affect the abnormal metabolism of this tissue.

In vitro: human osteoarthritic chondrocytes

Since the cartilage also demonstrates extracellular matrix remodeling during osteoarthritis and the ephrin system is known to control extracellular matrix, the next step was to explore the implication of the ephrin-B2/EphB4 system in the abnormal metabolic activities of human osteoarthritic chondrocytes [22]. Data showed for the first time that both ephrin-B2 and EphB4 are expressed and produced by adult chondrocytes, and that EphB4 production, but not that of ephrin-B2, is increased in osteoarthritic cartilage, indicating an imbalance in the equilibrium of this system. Treatment of osteoarthritic chondrocytes with ephrin-B2 positively impacted the abnormal metabolism of osteoarthritic cartilage by inhibiting important catabolic factors involved in this disease, as well as some of the IL-1β-stimulated protein production, at the same time as increasing anabolic activity. More specifically, ephrin-B2 treatment significantly inhibited the inflammatory factors IL-1β, IL-6, and protein-activated receptor-2 (PAR-2) as well as MMP-1, MMP-9 and MMP-13, and the IL-1β-induced IL-6, MMP-1 and MMP-13. It also up-regulated type II collagen expression.

Hence, in human osteoarthritic cartilage, ephrin-B2 treatment could act in two different ways: by limiting the matrix degradation via inhibition of the most important interleukins, MMPs and other catabolic factors involved in osteoarthritic cartilage breakdown and, at the same time, by promoting the production of type II collagen, the cartilage-specific macromolecule.

In vivo: transgenic mouse model in which osteoarthritis was induced

The above *in vitro* experiments suggest that by enhancing the activation of this ephrin system, a protective effect could be imparted on the structural changes of the articular tissues during osteoarthritis. Further *in vivo* studies were therefore essential. Thus, to further validate *in vivo* the protective effect of this ephrin system during osteoarthritis, we used a bone-specific EphB4 overexpressing mouse model, tg(transgenic)EphB4. This model was chosen since, as mentioned above, evidence suggests that the subchondral bone is an active component of the osteoarthritis process and its alterations may precede cartilage damage. Moreover, such a study could allow the ongoing discussion about the effect of subchondral bone remodeling during the osteoarthritis process to move forward. More specifically, this *in vivo* study could help to answer the question: Does prophylactic protection of the subchondral bone reduce the severity of osteoarthritis of other articular tissue lesions? Indeed, because the subchondral bone plate is in direct contact with the cartilage, the subchondral bone composition and structure would influence not only the mechanical properties of the overlying cartilage but also its metabolism in that, by cross-talk, it would provide factors that in turn would act on this latter tissue. Moreover, by protecting the subchondral bone, we hypothesized that this would also minimize the evolution of the disease severity in other articular tissues including the synovial membrane.

This tgEphB4 mouse model consists of an overexpression of EphB4 under the control of the type I collagen (*Col1*, main collagen type in bone) promoter [7]. Briefly, mouse *Ephb4* complementary DNA was subcloned upstream of the osteoblast-specific promoter region of the mouse proα1(1) collagen gene.

Data showed that the tgEphB4 mice have normal skeletal development and body weight [27]. To investigate the effect on osteoarthritis, the disease was induced surgically in the knee by the destabilization of the medial meniscus (DMM) [28] in 10-week-old male tgEphB4 mice. The articular tissues evaluated at 8 and 12 weeks post-surgery (when the lesions were at a moderate stage of the disease) revealed that the osteoarthritic (operated) tgEphB4 mice had better preserved subchondral bone morphology. Hence, there was a significant decrease in the subchondral bone plate thickness. Moreover, in osteoarthritic tgEphB4 mice, the TRAP levels were also significantly decreased compared to the control (wild type) mice, indicating a lower number of TRAP

positive cells. 3D rendering and reconstruction of the subchondral bone showed reduced bone volume and trabecular thickness in the osteoarthritic tgEphB4 mice. These findings indicate that during osteoarthritis, the overexpression of EphB4 in bone demonstrates a better preservation of the subchondral bone structure.

Importantly, these changes were associated with significantly less cartilage damage in the osteoarthritic tgEphB4 mice than in the controls, as seen by a decrease in histological signs and markers of cartilage degeneration, including a significant reduction in aggrecan and type II collagen degradation products, type X collagen, and fibril disorganization.

These *in vivo* findings further validate that by preserving the subchondral bone structure in order for it to be less prone to alterations such as microcracks and microfractures, diffusion of factors from the subchondral bone to the cartilage is also prevented.

However, because alterations in the synovial membrane, in addition to subchondral bone remodeling and cartilage destruction, are also a key phenotypic characteristic associated with the development of this disease, it was of importance to assess *in vivo* the effect of bone-specific EphB4 overexpression on the synovial membrane of this mouse model during osteoarthritis [29].

As synovial membrane also contains type I collagen, confirmation that the promoter used to generate the transgenic mice was strictly osteoblast-specific, was first done by determining the levels of EphB4 in the synovial membrane of the tgEphB4 and wild type mice. As expected, data revealed similar levels of EphB4 in these two mouse sets, indicating that if an effect was observed in the synovial membrane it would not be due to overexpression of EphB4 in this tissue and that the model targets only the bone.

Histomorphometric evaluation revealed a significantly reduced synovial membrane thickness in the osteoarthritic tgEphB4 mice compared to controls, suggesting a loss of integrity of the tissue. Confirming this assumption, evaluation of the angiogenesis factor VEGF showed markedly decreased levels in the osteoarthritic tgEphB4 mice. Moreover, data ascertained that there was no difference in cell proliferation between osteoarthritic tgEphB4 and control mice, suggesting that the higher synovial membrane thickness in the osteoarthritic wild type mice is associated with the presence of fibrosis. Indeed, data showed the presence of fibrin in the osteoarthritic control synovial membrane, whereas no zone of fibrin was found in the osteoarthritic tgEphB4 mice. In addition, factors related to fibrosis were also significantly decreased in the synovial membrane of the osteoarthritic tgEphB4 mice, including types I and III collagen, as well as classic fibrotic markers including connective growth factor (CTGF), α-smooth muscle actin (αSMA) and cartilage oligomeric matrix protein (COMP).

As previous studies have found that the mouse strain used for this model, the C57BL/6, exhibits spontaneous osteoarthritis during ageing, in which a high incidence of changes is found from about the 17th month of life [30], the effects of the integrity of the synovial membrane in the tgEphB4 mice were further investigated in 6- 12-, and 24-month-old mice that did not undergo DMM surgery, thus non-osteoarthritis-induced. Findings also suggested a protective effect of the bone-specific overexpression of EphB4 on the synovial membrane. Histologic evaluation revealed that at 24 months of age, the tgEphB4 mice had a decrease in the synovial thickness and lining layers, which reached statistical significance for the thickness.

These two studies provide evidence that the protection of the subchondral bone during the osteoarthritis process has an extended effect on other knee joint tissues, thus stressing the *in vivo* importance of subchondral bone remodeling as playing a key role in the genesis of this disease.

Conclusion

To summarize, these studies first showed that ephrin-B2 is essential for normal long bone growth and development, that the ephrin-B2/EphB4 system has direct (*in vitro* studies) beneficial effects on osteoarthritic cartilage and subchondral bone, and that EphB4 bone-specific overexpression prophylactically protects not only the subchondral bone from the disease alterations, but also the cartilage and the synovial membrane.

Finally, in the context of identifying new candidates for therapeutic avenues in the treatment of osteoarthritis, this ephrin system appears extremely attractive as a therapeutic approach in the development of a disease-modifying osteoarthritis drug.

The economic burden associated with osteoarthritis is substantial, incurring billions of dollars each year in direct and indirect costs. The disease often leads to joint replacement surgery: however, such surgery is not necessarily the answer to this disease, as pain has been reported to be the leading cause of dissatisfaction following surgery. Preservation of joint tissue structure is the key to curing the disease. Therefore, the ultimate goal of osteoarthritis treatment should be to target its cause by

preserving or improving the patient's joint structure by preventing the destruction of the articular tissues.

In recent years, attempts to find a cure have been disappointing, due in part to side effects or toxicity of the drug candidates studied. There is a serious need to identify therapeutic candidates that, in addition to preventing symptoms, will also arrest the disease progression, thereby preventing loss of joint function. The above-mentioned studies revealed that modulation of the activity of the ephrin-B2/EphB4 system in the knee joint tissues could be a frontrunner as a candidate for osteoarthritis treatment, as therapies having a global joint approach, i.e. an impact on many joint tissues, may prove to be the most successful and beneficial for the patient. In this line of thought, with the data on the ephrin-B2/EphB4 system providing a rationale and foundation to be targeted in new drug osteoarthritis development programs, it is interesting to note that approaches exist to increase the *in vivo* half-life of EphB4 [31], thus enhancing its pharmacokinetics. It is also of interest that the development of compounds/agents that can enhance the activity of ephrin-B2 will be significantly facilitated, as the structure of the complex formed by the binding of ephrin-B2 to its EphB4 receptors as well as the crystal architecture of ephrin-B2 have been reported [32, 33], which will greatly accelerate the specific structure-based protein engineering of compounds.

Conflicting interests

The authors have declared that no conflict of interests exist.

Author contributions

J.M-P., G.V-F., and J-P.P. drafted, revised, and approved the final version of the manuscript.

Abbreviations

Eph: erythropoietin-producing hepatocyte kinase; IL: interleukin; KO: knockout; MMP: metalloproteinase; RANKL: nuclear factor κB ligand; OPG: osteoprotegerin; Osx1: osterix-1; PI 3-kinase: phosphatidylinositol 3-kinase; PAR-2: protein-activated receptor-2; TRAP: tartrate resistant acid phosphatase; VEGF: vascular endothelial growth factor.

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