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Dysregulation and phenotypic modification of osteoarthritic osteoblast by Galectin-3: Identification of cellular ligands

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Résumé de la thèse

Introduction

La galectine-3 (gal-3) est une lectine qui appartient à la superfamille des galectines. Cette protéine soluble n'est pas glycosylée et a une masse d'environ 30 kDa. La caractéristique commune des galectines est la présence d'au moins un domaine de reconnaissance des structures osidiques (CRD). En plus de ce CRD, la gal-3 est composée d'un long domaine flexible qui a des acides aminés semblables à une chaine de collagène, et qui est important pour la multimérisation de la gal-3. Finalement un petit domaine N-terminal contenant des sites de phosphorylation (Ser^{6}, Ser^{12}) a été identifié. Le gène de la gal-3 (LGALS3) a été cartographié sur le chromosome 14 humain (14q21-22). Ce gène est organisé en 6 exons avec le codon de démarrage de la traduction dans l'exon 2, tandis que l'exon 3 code pour la séquence répétée et les exons 4, 5 and 6 pour le CRD. Il est maintenant admis que la galectine-3 puisse être sécrétée par ectocytose[1]. De nombreuses données de la littérature indiquent que les fonctions de la gal-3 dépendent de sa localisation subcellulaire. Elle peut être impliquée dans l'épissage alternatif, la différenciation cellulaire, l'apoptose et dans les interactions cellules-cellules.

Quoique découverte en premier dans les macrophages, la gal-3 est largement distribuée dans différents tissus. En ce qui concerne le squelette la gal-3 est retrouvée dans les ostéoblastes, les ostéoclastes, mais aussi les chondrocytes .

Concernant sa localisation extracellulaire, il a été montré que la gal-3 était plus abondante au sein de l'articulation lors de phases inflammatoires de l'arthrose (OA) et de la polyarthrite rhumatoïde. Plus récemment, une autre étude a montré que la gal-3 était plus abondante dans les liquides synoviaux de patients OA que chez des sujets sains. De plus, nous avons démontré que les chondrocytes humains répondaient à la gal-3 exogène en augmentant l'expression de l'agrécanase-2 (ADAMTS-5, A Disintegrin And Metalloproteinase with ThromboSpondin motifs) et de la stromélysine (MMP-3), enzymes impliquées dans la dégradation des protéoglycanes. La gal-3 inhibe fortement la production d'ostéocalcine par les ostéoblastes OA. Finalement une injection intra-articulaire de gal-3 chez la souris induit après 4 jours des altérations du cartilage et de l'os sous-chondral. **Ces résultats indiquent donc que des niveaux**

élevés de gal-3 intra-articulaire tels que l'on pourrait retrouver dans les phases inflammatoires de certaines maladies rhumatismales, auraient des effets délétères sur les tissus de l'articulation.

Parmi les différents tissus de l'articulation nous nous sommes intéressés à l'os sous-chondral qui est la partie osseuse juste en dessous du cartilage articulaire. Cette localisation permet des échanges privilégiés entre l'os et le cartilage et fait de cet os sous-chondral un élément important dans le développement de l'arthrose. En fait, l'os sous-chondral est en premier lieu une fine plaque d'os cortical. Le flux sanguin de l'os sous-chondral apporte, en situation physiologique, 50% des besoins en glucose, en oxygène et en eau aux couches profondes du cartilage. L'os sous-chondral est formé d'une matrice extracellulaire principalement formée de collagène de type I représentant 90% des protéines totales. La minéralisation des fibrilles de collagène et des autres protéines ostéoïdes, principalement des glycoprotéines et des protéoglycanes est formée de cristaux d'hydroxy-apatite. Les charges anioniques des ces protéines ostéoïdes joueraient un rôle important dans le processus de minéralisation grâce à leur grande capacité à complexer des ions cationiques. La cellule principale de l'os sous-chondral est l'ostéoblaste qui joue un rôle central dans la production qualitative et quantitative de la matrice ostéoïde. Ces cellules régulent aussi la différenciation des ostéoclastes qui sont les cellules capables de résorber l'os. Plusieurs études montrent que le collagène de type I et la phosphatase alcaline sont des marqueurs précoces de la différenciation des ostéoblastes tandis que l'ostéocalcine et la minéralisation sont des marqueurs des stades tardifs de cette différenciation. De nombreuses études ont démontré que l'os sous-chondral est le site actif de nombreux changements morphologiques qui peuvent être différents au cours de l'arthrose mais qui sont partie prenante du processus pathologique[2,5,52]. Ces changements induisent des processus biochimiques cellulaires anormaux qui sont la source d'altérations du tissu contribuant également à la dégradation du cartilage. Par exemple, l'os sous-chondral subit un remodelage avec une phase précoce de résorption puis une formation osseuse importante. Toutefois cet épaississement reflète un accroissement de la matrice ostéoïde et non une augmentation de la minéralisation.

Les données de la littérature suggèrent fortement un rôle délétère de la forme extracellulaire de gal-3, sécrétée par la membrane synoviale, dans la progression de l'OA. Toutefois les rôles exacts de cette lectine sur les différents tissus ne sont pas encore totalement élucidés. C'est pourquoi dans les études présentées dans ce mémoire,

nous sommes attachés à investiguer les rôles de gal-3 au niveau des ostéoblastes de l'os sous-chondral.

Objectifs

Comme mentionné précédemment, les symptômes dans l'arthrose peuvent être associés à des phases inflammatoires. La galectine-3 est un facteur inflammatoire qui a été détecté dans le tissu synovial et dans le liquide synovial lors d'inflammation. Ces faits ont permis de suggérer que la gal-3 pouvait participer soit à l'initiation soit à la progression de l'arthrose. D'ailleurs des effets délétères de celle-ci ont été reportés sur le cartilage chez la souris.

Des études ont montré que les ostéoblastes de patients arthrosiques pouvaient être classés en deux groupes en fonction de leur production de PGE_2 et ainsi dénommés « high » ou « low » producteurs. Ces études ont aussi montré que les niveaux élevés de PGE_2 étaient des régulateurs de la production d'interleukine 6 (IL-6) par ces ostéoblastes. Or il est connu que les PGE_2 et l'IL-6 sont impliquées dans la résorption osseuse. Toutefois, dans les stades tardifs de l'arthrose, l'os sous-chondral est sclérosé et non pas ostéoporotique, présentant toutefois une minéralisation anormale. C'est pourquoi, il est nécessaire d'investiguer si d'autres facteurs pourraient modifier le phénotype des ostéoblastes, particulièrement sous vitamine D_3, qui chez l'Homme est la molécule majeure du contrôle de la différenciation tardive des ostéoblastes. Par exemple des études précédentes ont montré que les ostéoblastes arthrosiques exprimés de forts niveaux de TGF-β1 et de molécules impliquées dans la signalisation Wnt. Notre groupe a montré que l'expression de la chaîne α1 du collagène dans les ostéoblastes arthrosiques humains est inhibée par la gal-3 en absence de vitamine D_3 tandis que celle de l'ostéocalcine est inhibée même en présence de vitamine D_3.

Parallèlement, des études récentes menées par notre groupe ont montré que le phénotype ostéoblastique pouvait être perturbé par la teneur en oxygène, c'est pourquoi nous envisageons aussi d'étudier si les variations de teneur en oxygène pouvaient avoir un effet sur les rôles de la gal-3 dans les ostéoblastes.

En résumé, peu d'études ont globalement été réalisées sur le rôle de la galectine-3 dans l'arthrose et encore moins sur le rôle de gal-3 sur les altérations de l'os sous-chondral d'où la nécessité de réaliser des études en prenant en compte les différents perturbations du phénotype des ostéoblastes.

Nos objectifs seront donc de :

D'étudier, dans un premier temps, les réponses des ostéoblastes arthrosiques à la 1,25(OH)$_2$ VitD$_3$ sur les facteurs anaboliques que sont le TGF-β1 et les molécules de la signalisation Wnt.

D'investiguer la modulation du phénotype des ostéoblastes arthrosiques et d'identifier les mécanismes cellulaires impliqués.

L'ensemble du projet est schématisé dans la figure 1.1.

Matériel & Méthodes

i) Rôle de la 1,25(OH)$_2$ VitD$_3$ sur l'expression du TGF-β1 et de molécules de la signalisation Wnt.

Des ostéoblastes humains OA en premier passage (n = 11) sont incubés en présence ou non de 1,25 dihydroxyvitamine D$_3$ (VitD$_3$, 50 nM) pendant 24 h. L'expression d'ostéocalcine, du facteur de croissance transformant 1 (TGF-β1), de la protéine 2 apparentée au « Dickkopf » (Dickkopf-related protein 2, DKK2), de la R-spondine 2 (Rspo2), de Wnt5b et du récepteur 1 apparenté à celui des LDL (low-density lipoprotein related-receptor 1, LRP1) a été analysée par PCR quantitative en temps réel.

ii) Rôle de la gal-3 dans la perturbation du phénotype ostéoblastique arthrosique

Le but de cette étude est d'analyser les effets combinés de la tension en oxygène et de gal-3 sur la régulation de la leptine une adipokine impliquée dans l'arthrose, et sur celles d'une métalloprotéase (MMP1), de la 25-hydroxy vitamine D$_3$ 1-alpha-hydroxylase **(**CYP27B1), enzyme activant localement la vitamine D$_3$ et de la gamma-glutamyl carboxylase (GGCX) intervenant dans la régulation de la minéralisation. Des ostéoblastes provenant d'os sous chondral de patients arthrosiques ont été incubés avec de la gal-3 (5 µg/ml) en présence d'une teneur en oxygène de 20% (normoxie) ou 2% (hypoxie) d'oxygène. L'expression des gènes a été analysée par RT-PCR quantitative et la production des protéines par ELISA. Les voies de

signalisation ont été examinées par western-blot et discriminées à l'aide d'inhibiteurs spécifiques.

iii) Les ligands de gal-3 à la surface des ostéoblastes.

Les cibles membranaires reconnues par gal-3 sur les ostéoblastes restent inconnues. Il faut donc les caractériser dans le but d'identifier le ligand de gal-3 responsable de son effet. Parmi différentes cibles, gal-3 se lie à l'intégrine β1. De plus une étude a montré qu'une diminution du niveau d'intégrine β1 soit par des siARN soit avec des anticorps bloquants abaissait la production d'ostéocalcine dans des cellules MG63. Donc nous émettons l'hypothèse que gal-3 pourrait agir, au moins partiellement, en bloquant l'intégrine β1 à la surface des ostéoblastes. En effet, nos données montrent que parmi les différentes cascades régulatrices induites par gal-3, la voie de la PI3-kinase apparait comme primordiale. Cette dernière peut être activée par les intégrines et plusieurs fonctions biologiques des ostéoblastes sont également régulées via la voie intégrine/PI3-kinase .

Identifications des ligands après western blot.

Des fractions enrichies en membranes plasmiques d'ostéoblastes seront solubilisées dans du PBS contenant 0,05% Tween 20 (T-PBS) et centrifugées afin d'éliminer les particules non dissoutes. Le surnageant sera alors concentré en acétone puis les protéines reprises en tampon Laemmli et déposées sur un gel de SDS-PAGE. Après migration, ces protéines seront transférées sur membranes de PVDF. Après blocage, la membrane sera incubée toute la nuit avec de la gal-3 couplée à de la digoxigénine (250 ng/µl) en tampon TBS, en présence ou non de lactose 300 mM. La présence de lactose servira à identifier des liaisons lectine-dépendantes. Le signal sera détecté par chimioluminescence, en présence de substrat ECL, à l'aide d'un anticorps secondaire dirigé contre la digoxigénine et couplé à une peroxidase.

Purification des cibles de gal-3 localisées à la membrane plasmique des ostéoblastes.

L'autre approche pour trouver les ligands de gal-3 présents à la surface des ostéoblastes consistera à utiliser une chromatographie d'affinité en couplant de la gal-3 sur une résine de Sepharose. Des fractions enrichies en membranes plasmiques d'ostéoblastes seront solubilisées dans du PBS contenant 0,05% Tween 20 (T-PBS) et centrifugées afin d'éliminer les particules non dissoutes. Le surnageant sera alors chargé sur la colonne de gal-3-Sepharose. Après plusieurs lavages les protéines ayant de l'affinité

pour gal-3 seront éluées en présence de lactose puisque la gal-3 reconnait les structures lactosidiques et N-acétyl lactosidiques. La fraction éluée sera analysée par SDS-PAGE colorée au nitrate d'argent puis ces composants identifiées par spectrométrie de masse MALDI TOF grâce à la plateforme protéomique de la fédération 3209.

iv) Rôle de la gal-3 sur la minéralisation

La sclérose observée dans la plaque sous-chondrale reflète une augmentation de la matrice ostéoïde (protéines de la matrice) et non pas une augmentation de la minéralisation. Au contraire cette matrice est moins bien minéralisée et ceci s'explique par une dysrégulation de la coordination de l'expression des gènes respectifs codant pour les chaînes α1 et α2 du collagène de type I et aussi par une production anormalement élevée d'ostéocalcine par les ostéoblastes OA comparativement aux ostéoblastes normaux, l'ostéocalcine étant connue pour inhiber la minéralisation[39]. Nous avons donc émis l'hypothèse qu'un traitement chronique d'une culture d'ostéoblastes par de la gal-3 devrait favoriser la minéralisation. Elle sera évaluée par la coloration au rouge alizarine après 28 jours de culture. La coloration sera ensuite extraite et quantifiée au spectrophotomètre à 425 nm. Des Saos-2 qui sont des cellules provenant d'un ostéosarcome humain serviront de contrôle positif de la minéralisation et seront cultivées dans les mêmes conditions mais pendant 21 jours seulement.

Résultats

Rôle de la 1,25(OH)$_2$ VitD$_3$ sur l'expression du TGF-β1 et de molécules de la signalisation Wnt.

Tous les spécimens ont répondu à la VitD$_3$ puisque l'expression d'ostéocalcine (OCN) est augmentée. Cependant deux populations ostéoblastes ont été discriminées. D'une part les « forts répondeurs » chez lesquels la stimulation d'OCN est supérieure à 100 fois (moyenne 881 fois, $p<0,01$, $n = 5$) et d'autre part les « faibles répondeurs » ayant une induction d'OCN en dessous de 100 fois (moyenne 47 fois, $p<0,01$, $n = 6$). En fait, les « forts répondeurs » sont caractérisés par une faible expression basale d'OCN. En comparant ces deux populations et en absence de VitD$_3$, les « forts répondeurs » expriment plus de TGF-β1 (15 fois, $p<0,001$), de DKK2 (2,5 fois, $p<0,002$) et de Wnt5b (5,5 fois, $p<0,003$), alors qu'aucune différence n'est retrouvée pour Rspo2 et

LRP1. La présence de $VitD_3$ accentue ce profil d'expression mais corrige le taux d'expression d'OCN et favorise l'expression des agonistes de la voie Wnt.

Nous avons identifiés deux populations d'ostéoblastes OA grâce à l'expression d'OCN. Dans les conditions basales, ces deux populations expriment de façon différentielle le TGF-β1, Wnt5b et DKK2, ce qui suggère une différenciation et un phénotype hétérogènes des ostéoblastes chez les patients OA.

Rôle de la gal-3 dans la perturbation du phénotype ostéoblastique arthrosique

La gal-3 stimule l'expression du gène de la collagénase 1 (*MMP1*) de 3 à 4 fois et de la production protéique (2,5 à 3,5 fois) en normoxie et hypoxie ($p<0,05$, n=6) comparé avec le contrôle. La gal-3 augmente l'expression et la quantité de leptine de 2,8 et 1,8 fois, en hypoxie ($p<0,05$, n=5). Les voies ERK1/2, p38 MAPK, PI3K et beta caténine sont activées par gal-3. Que ce soit en normoxie ou hypoxie, les voies de régulations principales sont p38 MAPK (inhibition 80% par SB203580 2 µM) et PI3K (inhibition 80% par LY294002 10 µM) pour MMP1 au niveau du messager et de la protéine ($p<0,05$, n=7). Pour la leptine, la voie ERK1/2 (inhibiteur : PD98059 5 µM) est également impliquée et le blocage de chacune de ces 3 voies inhibe la production de leptine à environ 90%.
La gal-3 stimule l'expression de la gamma-glutamyl carboxylase (GGCX, 3,4 et 1,6 fois) en normoxie et hypoxie, respectivement ($p<0,05$). La 25-hydroxy vitamine D_3 1-alpha-hydroxylase (CYP27B1) est stimulée en normoxie (4 fois) mais fortement inhibée 60% en hypoxie par la gal-3 ($p<0,05$). Que ce soit en normoxie ou hypoxie, les voies de régulation principales sont p38 MAPK et PI3K pour GGCX. CYP27B1 est différentiellement régulée avec l'implication des voies P38 et PI3K en normoxie et de toutes en hypoxie.

Les ligands de gal-3 à la surface des ostéoblastes.

En utilisant la technologie de reconnaissance lectinique sur western blot, nous avons pu montrer que la gal-3 se liait préférentiellement qu'avec une seule glycoprotéine de masse moléculaire apparente comprise entre 150 et 200 kDa, ce qui semblait étonnant. Cette liaison était cependant challengée par la présence de lactose ce qui semblait indiquer une reconnaissance lectinique spécifique. Cependant, nous avons rencontré

des problèmes de reproductibilité ce qui nous a orienté vers une autre technologie plus fiable.

La chromatographie d'affinité couplée à la spectrométrie de masse, a permis d'identifier une vingtaine de ligands potentiels dont 3 seront particulièrement ciblés pour leur rôle potentiel dans la maladie. Parmi ceux-ci nous nous sommes intéressés au CD44, 4F2hc et aux intégrines ß1 et ß5. En utilisant les siRNAs correspondants à ces ligands, nous avons démontré que la collagénase 1 induite par gal-3 était sous la dépendence de ces trois gènes même si SLC3A2 semble jouer un rôle prédominant ($p<0,05$, $n=12$). Contrairement aux autres ligands, le silencing de l'intégrine ß5 stimule la production de MMP1 que ce soit en absence ou en présence de gal-3. Concernant le mode de régulation de la leptine par ces quatre ligands de gal-3 nous montrent qu'elle est régulée uniquement par 4F2hc. Contrairement à MMP1, nous n'avons observé aucun phénomène de stimulation basale de la leptine en empêchant l'expression par siRNA d'un des ligands étudiés.

Rôle de la gal-3 sur la minéralisation

Que ce soit pour les ostéoblastes arthrosiques ou pour les Saos, nous avons montré que l'hypoxie diminuait la minéralisation. De la même façon la culture de ces cellules en présence de 50 nM de $VitD_3$ inhibait aussi cette minéralisation. L'utilisation de dexaméthasone comme contrôle positif favorisant la minéralisation n'a pas fonctionné de façon reproductible sur les ostéoblastes arthrosiques en normoxie alors qu'en hypoxie nous avons toujours observé une augmentation de la minéralisation avec cette molécule. Les Saos semblent insensibles à la dexaméthasone en ce qui concerne la minéralisation. Quel que soit le type cellulaire et quel quelles que soient les conditions de cultures l'ajout de galectine-3 n'a en rien perturbé la minéralisation.

Discussion

Durant l'arthrose, l'os sous-chondral se sclérose indiquant que de l'ostéogenèse se produit, bien qu'il y ait une hypominéralisation. Cependant il est suggéré dans la littérature que les ostéoblastes arthrosiques présenteraient une différenciation anormale avec notamment le TGF-β1 et des molécules intervenant dans la voie Wnt qui seraient impliqués. Nous avons pu identifier deux populations d'ostéoblastes. En fait, une population présentait un taux basal d'ostéocalcine élevé tandis que l'autre population avait un taux faible ce qui engendrait des fluctuations dans les réponses de ces deux

populations à la $VitD_3$. Ces niveaux de base d'ostéocalcine ont été attribués par des auteurs à un état de différenciation plus ou moins avancé des ostéoblastes. Les ostéoblastes les plus différenciés ont des niveaux d'ostéocalcine les plus élevés. Cela suggère donc que les ostéoblastes d'un patient arthrosique à un autre ne sont pas dans le même état de différenciation mais ceci nécessite de faire une étude sur une plus grande cohorte pour affirmer ce princeps. La différenciation moins aboutie de certains ostéoblastes pourrait être due à leur capacité à exprimer de forts taux de Wnt5b et peut-être d'autres agonistes de la voie Wnt. Cette hypothèse est d'autant plus plausible qu'en dépit de leur capacité à produire aussi plus de DKK2 qui est un antagoniste de la voie Wnt, le rapport Wnt5b/DKK2 varie de 1 à 2,5 entre les ostéoblastes fortement différenciés versus les ostéoblastes faiblement différenciés, favorisant ainsi l'effet de Wnt5b dans les ostéoblastes faiblement différenciés et donc sa capacité à inhiber une maturation de ces derniers. De plus, les ostéoblastes faiblement différenciés présentaient des taux de TGF-β1 élevés, ce qui est en accord avec la littérature où il est décrit que le TGF-β1 inhibait la différenciation tardive des ostéoblastes.

L'inhibition par la galectine-3 de l'ostéocalcine a été démontrée par notre groupe dans des études antérieures. Nos études dans ce travail montrent que malgré cette régulation de l'ostéocalcine, la galectine-3 n'est pas capable de moduler in vitro la minéralisation des ostéoblastes arthrosiques qui ont des problèmes intrinsèques de minéralisation mais aussi des cellules Saos qui malgré leur compétence à minéraliser sont également insensible à un rôle de la gal-3 dans ce domaine. On peut suggérer que, lors des cinétiques de minéralisation qui prennent plusieurs semaines, il puisse y avoir un phénomène d'échappement quant à la régulation de l'ostéocalcine par la gal-3.

Nos études développées dans ce travail de thèse démontrent que la gal-3 est aussi capable de stimuler la production de leptine en hypoxie. Nous avons récemment montré que l'hypoxie est un puissant stimulant de la production de leptine par les ostéoblastes. Or la leptine est associée à une aggravation de l'arthrose chez la souris et l'humain. Ceci suggère donc que lors des phases inflammatoires, la galectine-3 aurait également un rôle d'inhibition de la différenciation tardive des ostéoblastes. Quand on sait que les phases inflammatoires sont souvent associées à une hypoxie du liquide synovial ou que l'os sous-chondral arthrosique peut être soumis à de l'hypoxie, on imagine alors l'impact que peut avoir une forte sécrétion de gal-3 lors de ces phases inflammatoires. La stimulation de la leptine par la gal-3 est régulée par les 3 voies de signalisation

étudiées à savoir ERK1/2, p38 MAPK et PI3K. Contrairement aux résultats obtenus sur la régulation de l'ostéocalcine par la gal-3, on peut remarquer qu'en plus de la voie PI3K, les autres voies sont aussi impliquées. Cela dénote la complexité d'actions de la gal-3 sur un type cellulaire. Parmi les ligands étudiés, seul 4F2hc semble être en relation avec l'activation de l'expression de la leptine. Des études par d'autres groupes avaient montré avec d'autres types cellulaires que 4F2hc pouvait être un ligand cellulaire de gal-3 plus ou moins associé avec une intégrine. Parmi les 20 ligands identifiés avec la colonne de gal-3 Sepharose, dans la famille des sous unités β des intégrines, seules les sous unités β1 et β5 ont été révélées et celles-ci ont été ciblées par les siARN. Donc ici soit le 4F2hc peut agir seul, soit il peut être couplé avec des protéines non encore identifiées. Parallèlement à l'identification des sous unités β1 et β5, nous aurions pu aussi cibler les sous unités αv et α11 qui sont également retrouvées dans les 20 ligands s'accrochant sur la colonne de gal-3 Sepharose et qui doivent s'hétérodimériser avec les sous unités β, mentionnées ci-dessus.

Non seulement la galectine-3 modifie le phénotype des ostéoblastes mais elle stimule la production de la collagénase 1 (MMP1) qui est l'enzyme capable de dégrader la matrice ostéoïde. Ce type de régulation semble cellule spécifique puisque nos travaux antérieurs avaient montré que la MMP1 n'était pas stimulée dans les chondrocytes humains. Par contre, c'était la stromélysine 1 (MMP3) qui était stimulée. L'effet extracellulaire de gal-3 sur MMP1 n'avait d'ailleurs jusqu'à maintenant pas été montré. Par contre la littérature décrit dans deux publications différentes que l'expression de MMP1 pouvait être régulée au niveau du complexe protéique AP1 par la galectine-3 intracellulaire. Ceci soulève la question de savoir si la galectine-3 extracellulaire ne pourrait pas être internalisée et activer le gène *MMP1*. Cependant l'étude des ligands, comme on le verra plus loin, semble rejeter cette hypothèse. Contrairement à la leptine, MMP1 est régulée uniquement par deux voies à savoir p38 MAPK et PI3K que ce soit en normoxie ou hypoxie. Le silencing de chacun des ligands potentiels à savoir, CD44, 4F2hc et de l'intégrine β1 amoindri significativement la stimulation de MMP1 par gal-3. Ceci indique donc clairement que la stimulation de l'expression de MMP1 ne se produit pas à cause de l'internalisation de gal-3 car le silencing d'un seul de ces ligands n'aurait pas un impact aussi fort si un mécanisme d'internalisation puis d'activation existait. A l'inverse le silencing de l'intégrine β5 permet de stimuler

l'expression de MMP1 ce qui indique une régulation directe entre la reconnaissance de cette protéine avec l'environnement cellulaire et la régulation de MMP1.

Il a été décrit dans la littérature que les ostéoblastes avaient la possibilité de rendre active la 25 hydroxy $VitD_3$ grâce à leur hydroxylase CYP27B1. Nous avons étudié les effets de gal-3 sur cette enzyme et avons obtenus des résultats contrastés. En effet la gal-3 semble capable de stimuler CYP27B1 en normoxie mais l'inhibe en hypoxie. De même en utilisant les inhibiteurs des voies de signalisation activées par gal-3 nous avons observé que ces inhibiteurs empêchaient la stimulation de CYP27B1 en normoxie et au contraire prévenaient l'inhibition en hypoxie. Il semble donc que les trois voies soient impliquées que ce soit en normoxie ou hypoxie. Il est à noter cependant que l'expression de CYP27B1 est relativement faible dans les ostéoblastes arthrosiques humains puisqu'en PCR quantitative, nous avons retrouvé des Ct qui étaient supérieurs à 30. Il n'est donc pas impossible que les résultats obtenus dans ces expériences puissent être inexactes à cause des limites de sensibilités de la méthode. Comme nous l'avons mentionné plus haut, l'ostéocalcine est une protéine importante du métabolisme ostéoblastique. Son activité dépend de sa capacité à fixer les ions cationiques notamment le calcium grâce à la gamma carboxylation de résidus d'acide glutamique présents dans la séquence primaire de cette protéine. L'enzyme réalisant cette carboxylation est la GGCX. L'expression de cette enzyme est stimulée par gal-3 en normoxie et hypoxie. La régulation passe principalement par la voie PI3K en normoxie et par les voies PI3K et p38 MAPK en hypoxie, la voie ERK ayant un rôle mineur dans les deux conditions. Encore une fois ce ne sont que des mesures d'expression et non pas d'activité de l'enzyme mais il semblerait que gal-3 favoriserait la gamma carboxylation permettant donc à l'ostéocalcine d'avoir une conformation adéquate pour assurer son rôle. Ceci est étonnant car jusqu'à maintenant nous avons montré que gal-3 avait un rôle plutôt délétère sur le métabolisme osseux. On peut émettre l'hypothèse que les ostéoblastes mettent en place une sorte de rétrocontrôle afin de pouvoir réparer l'os une fois que les phases inflammatoires seront résorbées.

Conclusion

Nous avons identifié deux populations d'Obs OA grâce à l'expression d'OCN. Dans les conditions basales, ces deux populations expriment de façon différentielle le TGF-β1, Wnt5b et DKK2, ce qui suggère une différenciation et un phénotype hétérogènes des Obs chez les patients OA.

Globalement nos résultats confirment le rôle délétère de la gal-3 dans l'articulation lors d'inflammation puisqu'elle stimule la production de collagénase 1 impliquée dans la dégradation osseuse. De plus, elle accentue la perturbation phénotypique des ostéoblastes qui produisent plus de leptine lors d'épisodes hypoxiques. Bien que plusieurs ligands membranaires puissent médiater les effets de gal-3, 4F2hc semble jouer un rôle récurrent. Les voies de signalisation cellulaire ne sont pas identiques pour tous les gènes que nous avons étudiés.

Ces travaux montrent la complexité du rôle que peut avoir gal-3 dans l'articulation lors de phases inflammatoires. Pour contrecarrer, les effets de gal-3, il semble illusoire de cibler les voies de signalisation car la plupart des thérapeutiques ciblant les voies de signalisation cellulaire ont échoué, ces mécanismes étant ubiquitaires. Le ciblage de 4F2hc pourrait être intéressant semble concrètement très difficile à réaliser si on veut cibler les ostéoblastes. Il apparait donc que le plus facile est de bloquer les actions de la gal-3. Toutefois, l'élaboration d'inhibiteurs glycosidiques puissants et sélectifs de gal-3 reste un challenge à cause de la faible affinité de la protéine vis-à-vis des résidus glycosidiques et de l'homologie de séquence des acides aminés de toutes les galectines liant ses structures.

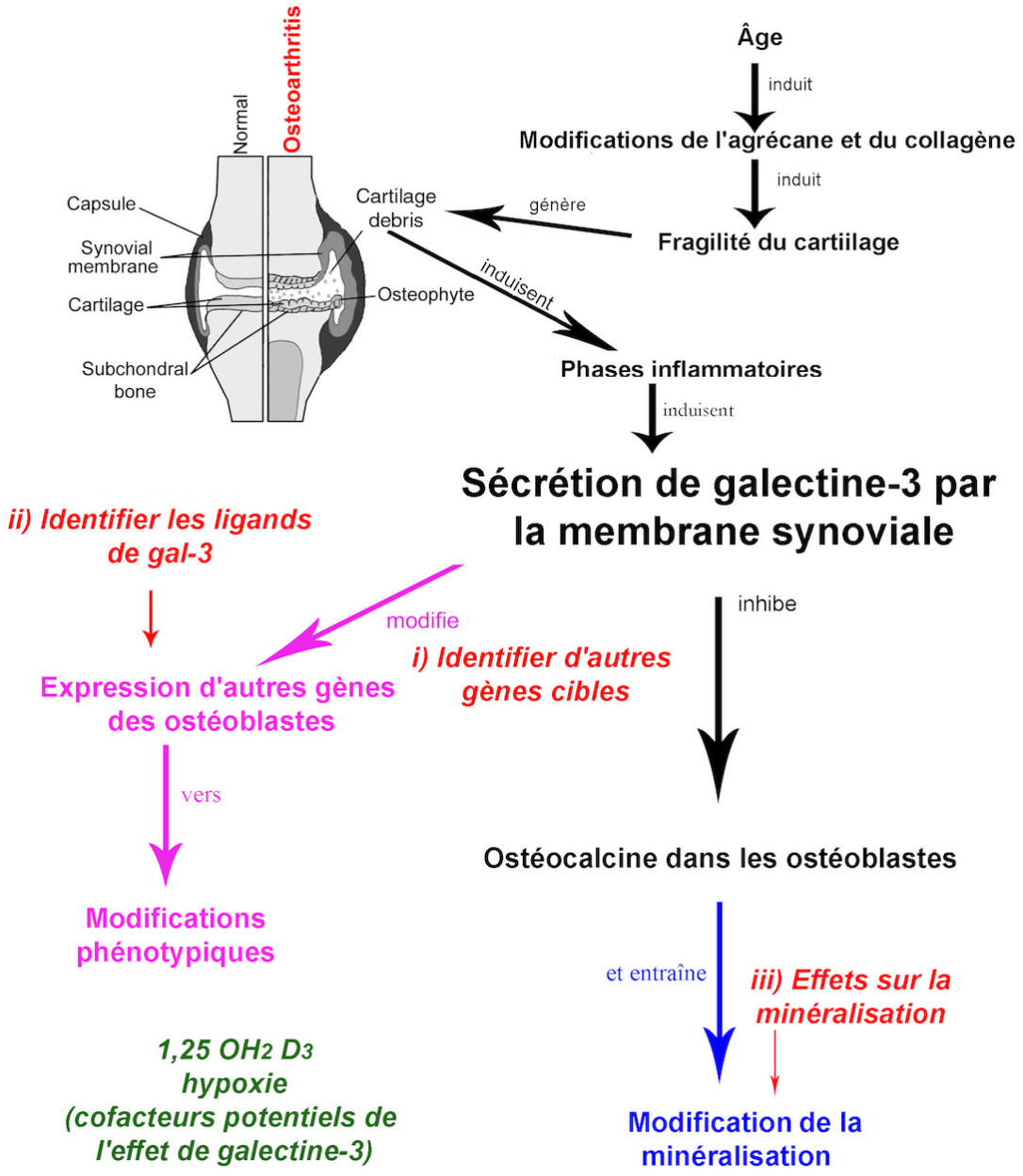

Figure 1. 1: Vue générale du projet.

List of Publications and Communications

List of publications

1. **Yong Hu**[*], Yang Liu[*], Daniel Lajeunesse, Didier Mainard, Jean-Yves Jouzeau and Pascal Reboul. Identification of two populations of osteoarthritic osteoblasts according to the 1,25$[OH]_2$ Vitamin D_3 potency to stimulate osteocalcin. ***Bio-Medical Materials and Engineering***. 2015 S103-S110.

2. **Yong Hu**, Mélissa Yéléhé Okouma, Korng Ea, Jean-Yves Jouzeau, Pascal Reboul. Galectin-3: a factor greatly involved in arthritis. (submitted in Joint Bone Spine)

3. **Yong Hu,** Arnaud Bianchi, Jean-Baptiste Vincourt, Patrick Netter, Didier Mainard, Jean-Yves Jouzeau, Pascal Reboul. Collagenase 1 and leptin are differently regulated by several galectin-3 ligands in human osteoarthritic osteoblasts (In preparation)

([*] These authors have contributed equally)

List of communications

Oral presentations:

1. **Y. Hu**, A. Bianchi, P. Netter *et al*. the regulation mechanism of leptin and collagenase I by galectin-3 in osteoarthritic osteoblasts in the presence of various oxygen tension. ***Annual conference《international scientific meeting of French arthroscopy society》***, December 3-6 2014 in Luxembourg.

2. **Y. Hu**, A. Bianchi, P. Netter *et al*. the regulation mechanism of leptin and collagenase I by galectin-3 in osteoarthritic osteoblasts in the presence of various oxygen tension. ***FR3209《international meeting of cell engineering and therapy》*** september 11 2014 in Nancy, France.

Poster communications:

1. **Y. Hu**, A. Bianchi, J.B. Vincourt *et al*. Galectin-3 induces regulation of collagenase 1 through mainly 4Fh2c in human osteoarthritis osteoblasts. ***3rd World Congress on Controversies, Debates & Consensus in Bone, Muscle & Joint Diseases (BMJD)***, april 24-26 2015 in Montreal, Canada.

2. **Y. Hu**, A. Bianchi, P. Netter *et al*. The regulation mechanism of collagenase 1 and leptin by galectin-3 in osteoarthritic osteoblasts in the presence of various oxygen tension. ***2nd national rheumatism scientific meeting of French rheumatism society***, December 5-6 2014 in Paris, France.

3. **Y. Hu**, A. Bianchi, P. Netter *et al*. Galectin-3 modulates gene expression in human osteoarthritic osteoblasts under normoxia and hypoxia. ***Scientific meeting of doctoral school*** april 01 2014 in Nancy, France.

4. **Y. Hu**, A. Bianchi, P. Netter *et al*. Galectin-3 modulates gene expression in human osteoarthritic osteoblasts under normoxia and hypoxia. ***9th international meeting of biomedical research*** March 21 2014 in Nancy, France.

5. **Y. Hu**, A. Bianchi, P. Netter *et al*. Galectin-3 modulates gene expression in human osteoarthritic osteoblasts under normoxia and hypoxia. ***8th international scientific meeting of cartilage engineering*** September 26-27 2013 in Nancy, France.

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Abbreviations

A Disintegrin And Metalloproteinase with Thrombospondin Motifs (ADAMTS)

Alkaline Phosphatase (ALP)

Aminopeptidase N (CD13)

Anterior Cruciate Ligament (ACL)

Body Mass Index (BMI)

Bone Marrow Edema-like Lesions (BMELs)

Bone Morphogenetic Proteins (BMPs)

Carbohydrate Recognition Domain (CRD)

c-Jun N-terminal Kinases (JNKs)

Damage Associated Molecular Patterns (DAMPs)

Decay-Accelerating Factor (DAF)

Dickkopf (DKK)

Dipeptidyl Peptidase-4 (CD26),

Extracellular Regulated Kinases (ERKs)

Fibroblast Growth Factors (FGFs)

Formyl-methionyl-leucyl-phenylalanine (fMLP)

Frizzled-Related Protein 3 (sFRP3 or Frzb)

Heart Failure (HF)

Heat Shock Proteins (HSPs)

Hepatocyte Growth Factor (HGF)

Hyaluronan acid (HA)

Hypoxia Inducible Factor (HIF-1)

Insulin-like Growth Factor-1 (IGF-1)

InterCellular Adhesion Molecule (ICAM)-1

Interferon γ (IFNγ)

Interleukin (IL)-1β

Lipopolysaccharide (LPS)

Macrophage Colony-Stimulating Factor (M-CSF)

Magnetic Resonance Imaging (MRI)

Mesenchymal Stem Cells (MSC)

Metalloproteinases (MMPs)

Mitogen-Activated Protein Kinases (MAPK)

Non Specific Esterase (NSE)

N-terminal domain (ND)

N-Terminal pro Brain Natriuretic Peptide (NT-ProBNP)

Nuclear-Factor κB (NF-κB)

Osteoarthritis (OA)

Osteoprotegerin (OPG)

ParaThyroid Hormone (PTH)

Pathogen-Associated Molecular Patterns (PAMPs),

Pattern Recognition Receptors (PPRs)

Prostaglandin E2 (PGE2)

Prostatic Acid Phosphatase (PAP),

Reactive Oxygen Species (ROS)

Receptor Activator of NF-kB Ligand (RANKL)

Receptor of Advanced Glycation End-products (RAGE)

Rheumatoid Arthritis (RA)

SubChondral Bone (SCB)

TCR—T cell receptor

Toll-Like receptors (TLRs)

Transforming Growth Factor-β (TGF-β)

Tumor Necrosis Factor (TNF)

Type IIA procollagen (COL2A)

UDP Glucose Dehydrogenase (UDPGD)

Vascular Cell Adhesion Molecule (VCAM)-1

Vascular Endothelial Growth Factor (VEGF)

Wingless Type (WNT)

WNT-Inducible Signaling Pathway Protein 1 (WISP1)

World Health Organization (WHO)

Zinc Alpha-2-Glycoprotein (ZAG),

β1, 6-N-acetylglucosaminyltransferase V (Mgat5)

CHAPTER I

INTRODUCTION

1 Osteoarthritis

1.1 Introduction

Osteoarthritis (OA) is the most common joint disease, which has already become a leading cause of disability in older adults. Considered as an important burden in global economic cost, OA was characterized by cartilage degradation, bone remodeling and synovial inflammation. During OA, the interplay cross-talk between overlying cartilage and subchondral bone is regarded as crucial. The joint tissue remodeling can also be initiated or triggered by a host of pro-inflammatory factors. Among them, a β-galactoside binding protein, galectin-3 (Gal-3) may play a role. In our research, we will focus on Gal-3 and its effects on phenotype modifications of OA osteoblasts, the mechanism will be investigated as well.

1.2 Osteoarthritis history

From the times of Hippocrates until only the past 250 years, various historical reports indicate that all forms of chronic OA are manifestations of gout. A hint of discrimination was illustrated in the notice of Heberden on *Digitorum Nodi* in 1782. However, the first description was published in 1802. William Heberden the Elder made a clear explanation of the disease, and claimed that it has no relation with gout[6]. The disease was found not only in fingers, but also in other articulations, particularly in hip. John Haygarth has proposed a magnificent description of the multi-articular disease, which was almost identical with what we see at present. Though the exact origin of concept in the description of hip OA was obscure, most of the people accept what was firstly presented by Robert Adams in literature in 1831. This concept was then distinguished from rheumatoid polyarthritis by Robert Smith in 1835. The definition of the disease progresses from arthritis deformans in 1869 to osteoarthritis in 1890 and finally authoritatively in a separated form, with its own special pathology.

1.3 Osteoarthritis epidemiology

1.3.1 Definition

OA, which is also known as a degenerative joint disease, is the clinical and pathological outcome of a range of disorders that result in structural and functional failure of synovial joints. It is characterized by focal areas of articular cartilage loss within synovial joints associated with bone hypertrophy (osteophytes and subchondral bone sclerosis) and capsule thickening. OA can be found in any joint, but most common located in hip and knee. The preferred diagnosis of OA should be made with considering the clinical symptoms, physical examination and X-ray findings, as either finding alone leads to overestimates.

1.3.2 Public health epidemiology

OA is the most common form of arthritis, it is considered as one of the most causal diagnosis in general practice[7]. Around 10% of the world's population age 60 years or older were affected by OA[8]. In 1995, Oliveria SA *et al*. have demonstrated that age- and gender-standardized incidence rates increase sharply after age 50 years and begin to decrease after age 70 years (Figure 1.1.1)[9].

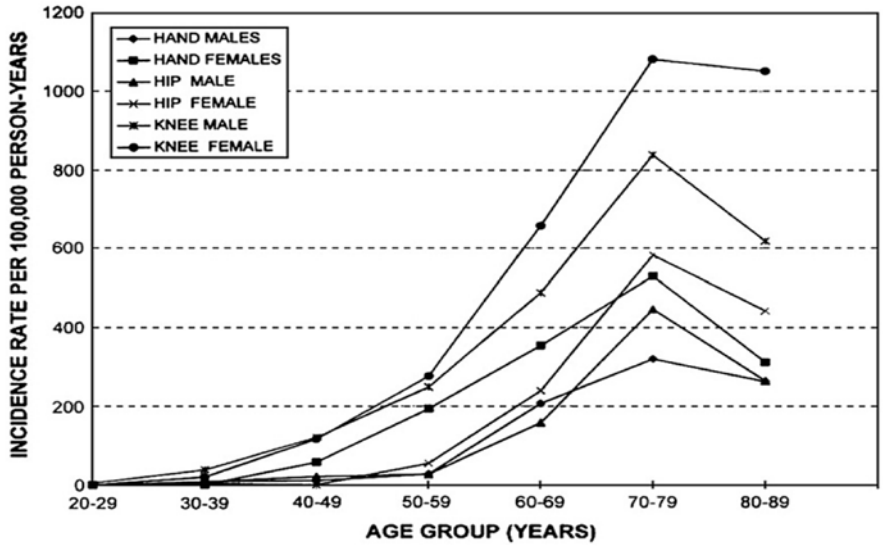

Figure 1.1. 1: Incidence of OA of hand, hip, and knee in a community health plan, 1991 to 1992, by age and gender. (Oliveria SA *et al*. 1995)

From 2010 to 2012, an estimated 52.5 million adults in the United States suffered from arthritis, 49.7% of adults 65 years or older are reported an arthritis diagnosis[10]. The number increased by 2.5 million than that reported in 2009[11,12].

In France, research from le Pen in 2005 reported that the prevalence of OA is about 17%, with 9-10 million patients, half of them with symptoms[13]. More recently, Guillemin *et al*. estimated the prevalence of symptomatic knee and hip OA in France, a two-phase population-based survey was conducted in six regions from 2007 to 2009. They demonstrated that the hip and knee standardized prevalence was 1.9% and 4.7% for men and 2.5% and 6.6% for women, respectively. The conclusion was also made that in France, the disease increased with age and is greater among women above the age of 50[14].

Medical Services Plan in British Columbia, Canada has conducted a survey. The survey included 4 million people to health professionals and hospital admissions showed that the overall prevalence of OA in 2001 was 10.8%, women occupied a predominant ratio regardless the age groups. In addition, above 30% of people suffered from OA in the age group between 70 and 74 years[15].

To evaluate the prevalence of the knee OA and associated factors, a survey covering 2093 residents aged 40 years or above based on community was carried out in Shanghai (People's Republic of China). In the community, the estimated prevalence of symptomatic knee OA was 7.2% and 37.4% for the asymptomatic one. Similar to that reported by others, more women suffered from OA than men and the prevalence of symptomatic OA increased with age[16].

Furthermore, not only gender and country, but also the ethnic group and geographic location could contribute to the OA epidemiology. A report was presented to estimate knee OA in seven regions of the world in 2000 (Figure 1.1.2). Combined with several other studies, they showed that African-American women are more liable to knee OA than white women[17,18]. On the other hand, hip OA are more common in European whites than in Jamaican blacks[19], African blacks[20], or Chinese[21]. In general, OA is more prevalent in Europe and the USA than in other parts of the world.

1.3.3 Society burden of OA

The costs of OA, rheumatoid arthritis (RA), osteoporosis and low back pain are major components of muscoskeletal burden, among which OA was considered to play a

dominant role[22,23]. It was estimated that OA will be the fourth leading cause of disability by the year 2020 due to the increase of life expectancy and aging populations[22].

The society burden of OA can be divided into two parts, direct costs and indirect costs. The former comprises the cost associated with drugs, medical care, surgery (total joint arthroplasty), hospitals, research, pensions and benefits. The latter can be described as premature mortality and chronic and short-term disability. The burden of a disease is related to not only its prevalence, but also the costs of the disease in the health care system of a country. In 1990, OA was estimated to be eighth leading no-fatal burden disease.

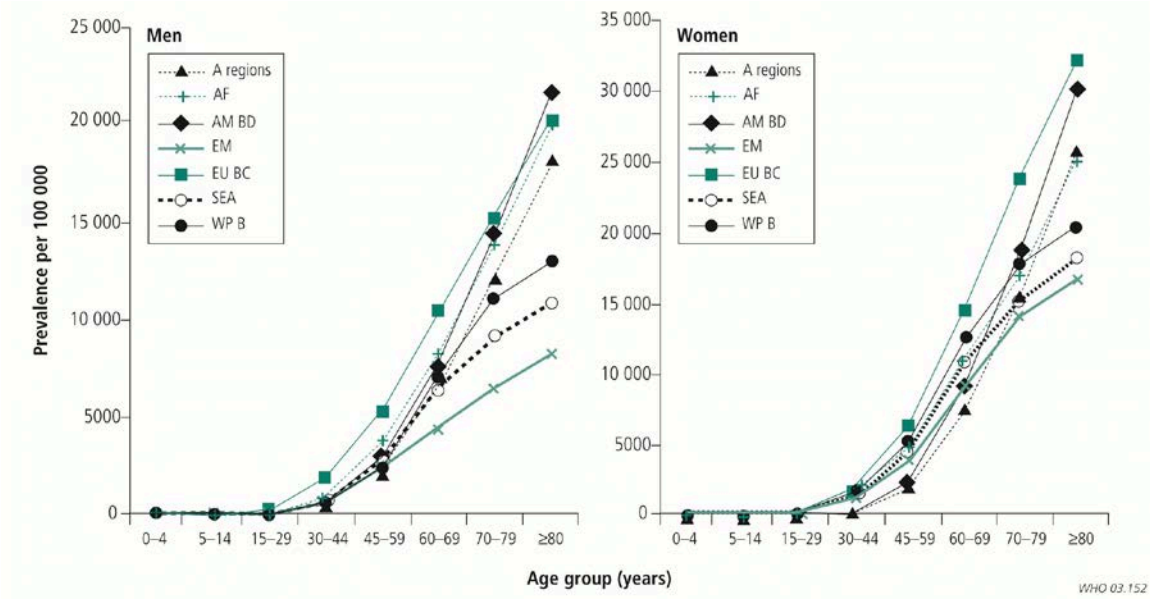

Figure 1.1. 2: Prevalence of osteoarthritis of the knee, by age group, gender, and region in 2000. A regions = developed countries in North America, Western Europe, Japan, Australia, and New Zealand. AF = countries in sub-Saharan Africa. AM BD = developing countries in the Americas. EM = countries in the Eastern Mediterranean and North African regions. EU BC = developing countries in Europe. SEA = countries in South-east Asia. WP B = countries in the Western pacific region (A D. Woolf *et al*. 2003).

In the United States, the total annual costs of OA for every patient were 5700 dollars[24]. Average direct costs of OA were 2600 dollars per year for one patient[25]. In 2009, estimated costs due to hospital expenditures of total knee and hip joint replacements, were $28.5 billion and $13.7 billion, respectively[26]. The job related OA costs were $3.4 to $13.2 billion per year[27].

In France, the society burden of OA has been examined early in 1993 and estimated to about 1 billion euros per year, including direct and indirect costs[28]. Ten years later, a

study was carried out to compare the change in the OA costs[13]. A schema was drawn to describe the distribution of OA costs (Figure 1.1.3), and a table with compared changes in OA financial costs between 1993 and 2003 was made (Table 1.1.1). Over the ten years, the population of patients with symptomatic OA increased by 54% in France, while in the same period, OA costs increased almost 3 times. Obviously, two third of the OA costs have been occupied by hospital admissions and medications. A more recent report presented in an international congress in 2012, demonstrated that annual costs for every French patient by general practitioner were 755 euros in 2010. The direct cost in treating OA per year has increased to 3 billion euros[29].

With the increase of life expectancy and ageing populations, the burden will be gigantic in developing countries, where the access to arthroplasty and joint replacement is not yet readily available[22]. Moreover, the burden of OA cannot be evaluated exactly, as several components of OA care are difficult to be estimated, such as outpatient investigations, treatments for adverse events related to surgery, loss of productivity and so on. Furthermore, with increasing number of primary arthroplasty, more arthroplasty revisions will be carried out. Correspondingly, during the arthroplasty revision, more blood loss, longer operation time, accompany with higher risk of cardiovascular adverse effects or accidents should be considered as the extra burden of OA independent of direct costs[13].

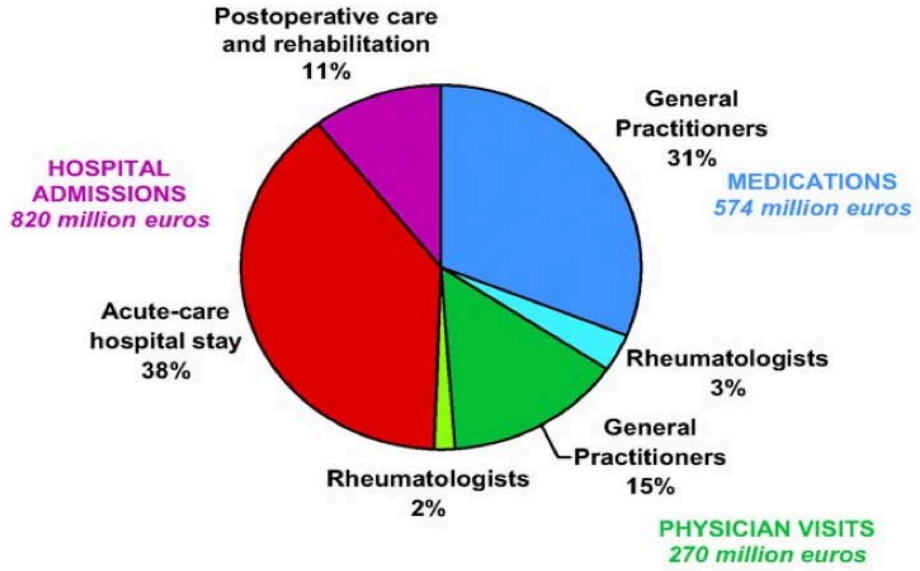

Figure 1.1. 3: Distribution of costs for OA (absolute numbers are annual costs). (C. Le Pen *et al*. 2005)

Table 1.1. 1 Changes in financial costs of osteoarthritis between 1993 and 2003. (C. Le Pen *et al*. 2005)

	1993 study by Levy et al.	2003 COART France study	Change (%)
Patients with symptomatic osteoarthritis	3 million	4.6 million	+ 53
Physician visits			+ 54
Number	8.7 million	13.4 million	
Cost, €	145 million	270 million	
Medications			+ 28
Number of prescriptions	14 million	18 million	
Cost, €	150 million	574 million	
Hospital admissions			+ 37
Number	93.000	127.000	
Cost, €	270 million	820 million	
Annual cost/patient			
Admitted	3425	5 800	
Not admitted	98	181	
Direct medical costs	641 million	1 644 million	

1.4 OA manifestation and diagnosis

Most people with OA seek for medical attention because of joint pain, which is aggravated by activity and relieved by rest. The pain will become constant and even awaking patients during nights in more advanced OA. The association between pain and weather changes such as temperature drop is also complained by OA patients[30]. The origin of pain is commonly not considered as coming from the cartilage due to its aneural character but other joint tissues such as synovium, subchondral bone, periosteum and joint capsule are suggested contribute to pain due to their innervated structures.

The other feeling in limiting daily activities described by patients is morning stiffness. Unlike RA, the OA stiffness sustains no longer than 30 minutes. Moreover, after a period of rest during the day, patients feel the stiffness again which disappears after moving but accompanied with more pain, a phenomenon called gelling[31,32].

Joint swelling and tenderness, crepitus and muscle atrophy are also present in many OA patients. The joint swelling derived from the accumulation of synovial fluid, or from bone deformity such as osteophyte formation, even indirectly from the bone marrow lesions represented edema[33]. The bony prominence can be palpable due to the formed osteophyte, joint subluxation and deformity such as varus and valgus will be present in advanced OA stage[34,35]. During the OA progress, patients are more and more liable to disuse the joint because of pain and other reasons, which leads to the atrophy of the surrounding muscles.

The diagnosis of OA depends on the clinical symptoms, physical examination and radiological features. Though the symptoms of patients are diverse, they are indispensible for OA diagnosis. The most important evidence is pain complained by patients. Only if the attention is paid on the pain origin, correlated examinations will be applied to diagnose OA. However, the threshold of pain in some patients is much higher. Therefore, they do not feel pain even though OA diagnosis is concluded by other symptoms and clinical examinations. Based on the symptom history, the physicians firstly make towards to physical examination including the measurement of joint motion range. During OA, especially in the late stage, the joint motion range will be limited obviously compared with normal. As for radiological examination, the X-ray is suggested as the most economic and effective method to diagnose OA (Figure 1.1.4), while CT and MRI are tended to be more applied in the early OA stage or in distinguish from other etiologies such as meniscal injury[32,36]. Besides them, laboratory tests are not necessary but can be helpful for the diagnosis, such as white blood cells count is usually less than 500 cells/mm^3 (0.5*10^9/L), and mainly presented by mononuclear cells[31]. It seems that the laboratory tests like immunological test are more in favor of the diagnosis of other arthritis such as RA.

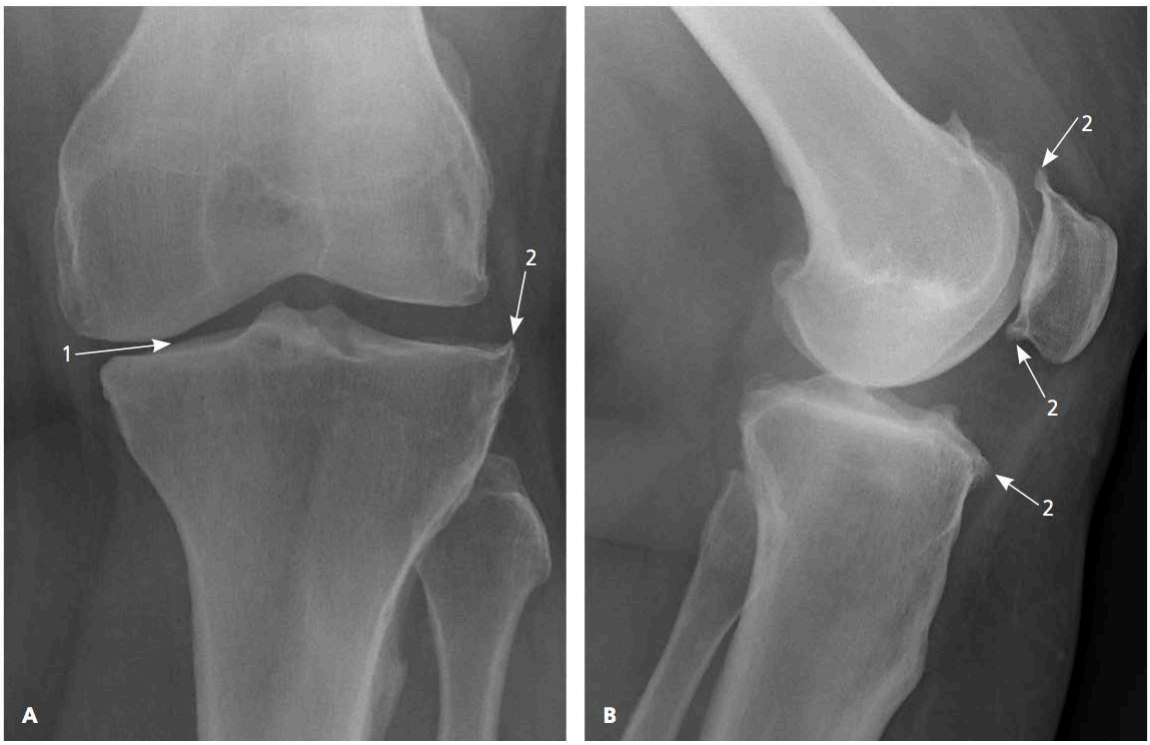

Figure 1.1. 4: Radiograph of the knee in (A) anteroposterior and (B) lateral views showing (1) joint space narrowing and (2) osteophyte formation. (Sinusas, K *et al*. 2012)

1.5 OA Risk factors

The etiology of OA has been considered as a complex process, which deserves to long-term investigation. A wide range of systemic, genetic, environmental and biomechanical factors are demonstrated, some of them being modifiable (Figure 1.1.5). However, the real mechanism of interplay among various factors contributing to the development of the disease has not been clearly elucidated yet.

1.5.1 Person-level risk factors

1.5.1.1 Age

With the rapid increase in the number of elderly people, the incidence of OA rises sharply. It is rarely that people under the age of 45 years suffered from OA, while nearly 60% of people over the age of 75 years are affected[9,37]. Known as one of the strongest predictors of OA, the relationship between age and OA deserves much more attention.

The association between OA and age is not fully understood, it is maybe a result of the cumulative joint damages over the life course coupled with reduced capacity for tissue repair[38]. This decreased ability of cells is due, at least in part, to be unable to respond properly to growth factor stimulation. For instance, chondrocytes in aged and OA cartilage are less responsive to transforming growth factor-β (TGF-β) and insulin-like growth factor-1 (IGF-1)[39].

Recently, more and more basic researches start to shed light on the aging changes in cells and tissues of older adults. Cells in elders were reported to exhibit a so called senescence-associated secretory phenotype, consisting in the production of various cytokines and matrix metalloproteinases (MMPs) similar to those found in OA joint tissues[40]. The special phenotype may be related to the induction of reactive oxygen species (ROS), which will induce cell death[41]. The mitochondria are an important source of ROS in cells, and mitochondrial dysfunction is thought to play a role in age-related diseases including OA (Figure 1.1.6). Except for cell death, the mitochondrial ROS act as signaling molecules and promote expression of pro-inflammatory cytokines[42]. Excessive levels of ROS inhibit the activation of the

IRS-1-PI-3kinase-AKT signaling pathway through oxidize low-density lipoprotein, leading to reduction of matrix synthesis. At the same time, the ERK MAP kinase is activated by ROS to suppress the expression of aggrecan, type II collagen and Sox-9 in chondrocyte[43]. Another feature is a loss of autophagy with age, represented by decreased autophagy markers in OA chondrocytes and related increase of cell apoptosis[44]. However, the connection between the age-related changes and the propensity for OA to occur in older adults is still missing.

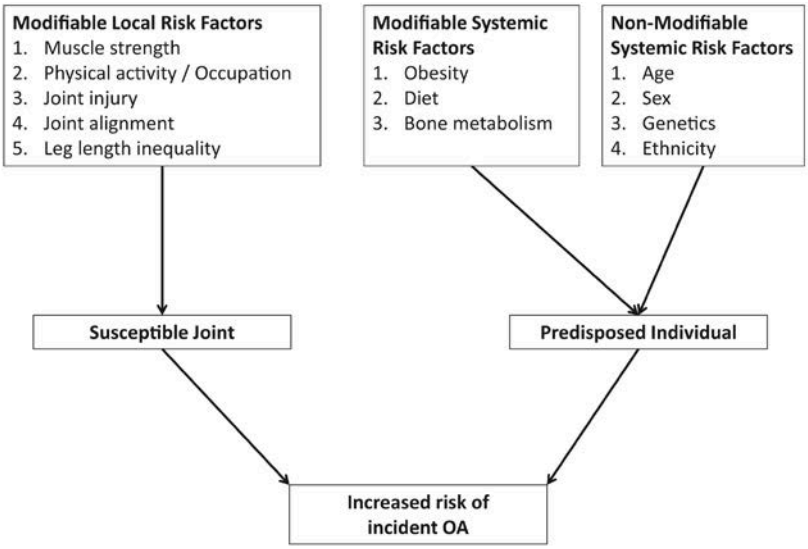

Figure 1.1. 5: Potential risk factors for susceptibility to OA incidence, each with differing degrees to support their association. (V.L. Johnson *et al*. 2014)

1.5.1.2 Gender

Female gender was considered as one of the main factors associated with onset of knee pain. A systematic review and meta-analysis has screened 46 studies distributed in 6554 articles, eleven cohort studies of them assessed female gender as a potential risk factor. There was consistent evidence that females were associated with higher prevalence and severity of OA[45]. Furthermore, female to male ratio differs across joint sites, hand, foot and knee OA was more often present in women. One of the possible explanations for gender difference is estrogen deficiency, based on the fact that the incidence of OA increases at the time of menopause[46]. A review of the literature on the role of estrogen in OA concluded that there was a weak protective effect of estrogen replacement therapy in the development of hip and knee OA but not for hand OA[47]. However, conflict data always exist on the relation between estrogen levels and the risk

of OA. On the other side, women may also have less volume of knee cartilage than men, whether this could contribute to accelerated loss of cartilage is still obscure[7,48].

1.5.1.3 Genetic factors

Results from several studies have shown that OA is inherited and may vary by joint site. The heritable component of OA has been estimated to be 40% to 65%, and stronger for hand and hip OA than for knee OA[38,49-52]. Despite extensive efforts, only GDF5 (a bone morphogenetic protein expressed in articular and skeletal structures), chromosome 7q22 and MCF2L have been associated with OA at genome-wide significance levels nowadays[53,54,55]. However, a recent large genome-wide association study from the arcOGEN Consortium has identified 5 new candidates related with OA, distributed in chromosome 3, 6, 9, 12 respectively[56].

1.5.1.4 Obesity

Obesity and overweight have long been recognized as potent risk factors for OA. As obesity is increasing in prevalence, more individuals will be affected by knee OA in the future. It is also associated with increased incidence of major diseases such as diabetes mellitus, cardiovascular disease, asthma, and most types of cancer[57].

Body mass index (BMI) is the most frequently used tool in the diagnosis of obesity, calculated as a patient's weight measured in kilograms divided by the height in meters squared (Table 1.1.2). It has the advantage that a subject's height and weight are easy and inexpensive to measure[58]. The World Health Organization (WHO) defined overweight as a BMI between 25.0 and 29.9 kg/m^2, and obesity as a BMI $\geq$ 30.0 kg/m^2. Compared with those of normal weight, overweight (not obese) will account for more than twice chances in developing OA, the risk will increase to almost 3 times if counted people who were obese or overweight[59]. On the other side, a weight reduction by 5 kg will decrease the risk of the development of knee OA by 50%[60,61]. The relationship between body weight and knee OA differs to that for hip or hand OA, regardless of an association always in presence.

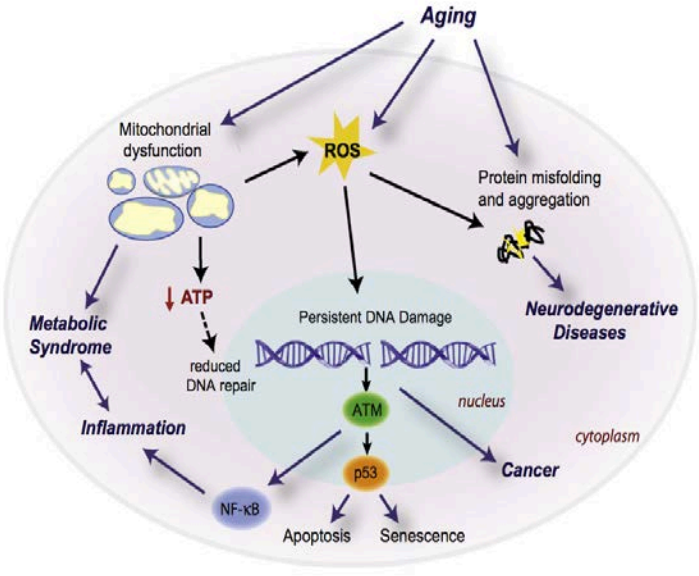

Figure 1.1. 6: Age-Related Stress and Disease Aging is associated with mitochondrial dysfunction, leading to reduced respiratory metabolism and increased generation of reactive oxygen species (ROS). (Haigis MC *et al*. 2010)

In the last decade, a low grade inflammatory state in the organism caused by obesity was proposed to result in direct pathologic effects on the musculoskeletal system, which was confirmed by elevated inflammatory markers, such as high-sensitivity C-reactive protein, increase of the erythrocyte sedimentation rate and elevation of interleukin-6 and tumor necrosis factor (TNF)[62]. During OA, a variety of pro-inflammatory cytokines named adipokines are also secreted by the excess mass of adipose tissue, among which, leptin is supposed to play a vital role.

Table 1.1. 2 International Classification of Adult Underweight, Overweight, and Obesity Based on Body Mass Index (RC Koonce, 2013)

Classification	Body Mass Index (Kg/m^2)
Underweight	<18.5
Normal range	18.5-24.9
Overweight	25.0-29.9
Obese	≥30.0

Leptin, a 16-kDa protein encoded by the obese gene, is the archetypal adipokine[57]. As its name suggests, leptin served to diminish food intake and increase energy expenditure at hypothalamic regulatory areas. The binding of leptin to hypothalamic leptin receptor (Ob-R) will switch on the JAKs and SRC family kinases SFKs,

following the phosphorylation of STAT proteins and other downstream targets. In obesity, those responses are abnormal, such as decreased receptor expression, increased expression of negative regulators of leptin signaling. Thereafter, the phosphorylation of STAT decreased, which indicates hypothalamic leptin resistance in obese patients[63].

Cell response to leptin in cartilage seems to be not the same as in hypothalamus. In OA articular cartilage, induction of leptin expression was found in chondrocytes accompanied with an increased level in synovial fluid[64-66]. In animal models, Dumond *et al*. reported that leptin injection into the rat knee increases the cartilage destruction, accompanied with the synthesis of IGF-1, TGF-β and leptin itself[64]. Vuolteenaho *et al*. described the link between obesity-related OA and leptin as shown in Figure 1.1.7. In this figure, they proposed that the leptin signaling in obese patients is enhanced by both the decrease of soluble ObR and SOCS-3, which are negative regulators of leptin responses. This triggers production of pro-inflammatory and cartilage-destructing factors[67]. In conclusion, a high level of leptin in synovial fluid of obese patients associated with cartilage destruction hints its role in OA pathogenesis.

Obesity is a modifiable factor that could be reduced or eliminated by education and support/guidance given by health-care professionals[68]. Much more attention should be paid to the increase epidemic of obesity, as which leads to an augmentation of OA incidence.

1.5.1.5 Other person-level risk factors

Other environmental factors, such as alcohol consumption, smoking and nutrition, have also been examined in their contributions to OA. No relation between alcohol intake and OA was found. Interestingly, smokers show a slightly lower risk of OA than non-smokers. Animal studies have emphasized the role of early life nutrition in the contribution to OA but this theory has not been verified in human yet. Vitamin D predicts the risk of osteoporosis, but its relation with bone mineral density is controversial[69]. Vitamin D deficiency is also related to rickets and osteomalacia. However, studies of the relationship between Vitamin D and OA have been conflicting. It has been postulated that low levels of Vitamin D may increase the incidence and progression of knee and hip OA[70-72]. Despite this, a recent randomized controlled trial of the effects of Vitamin D on knee OA demonstrated an unbeneficial effect on cartilage

loss by magnetic resonance imaging (MRI)[73]. In Framingham study, no relation was found between intake of Vitamin C and risk of incident OA[72]. Conversely, a cohort of healthy and middle-aged individuals who took dietary food rich in Vitamin C for 10 years, less cartilage loss and lower frequency of subchondral bone pathology was reported[74]. Similarly, Vitamin K is suggested to be involved in OA progression, as it reduces the prevalence ratio for OA due to its potential bone and cartilage effects[75,76].

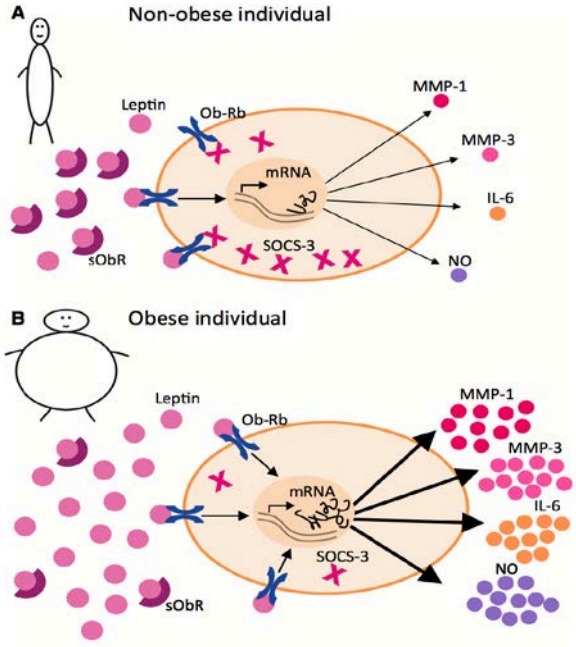

Figure 1.1. 7: Leptin: a factor of obesity-related osteoarthritis (OA). (K Vuolteenaho *et al*. 2014)

1.5.2 Joint-level risk factors

Biomechanical factors are also tightly associated with OA, such as reduced muscle strength, repetitive joint use, joint injury earlier in life, alignment and so on. These biomechanical factors are believed to act as an integrated complex, instead of acting alone respectively.

1.5.2.1 Reduced muscle strength

Quadriceps femoris is the primary antigravity muscle of the lower limb, through absorbing limb loading and providing dynamic joint stability. It plays a key role during ambulatory, functional and sport activities. Obviously, in case of reduced strength of quadriceps femoris, knee has to bear more loading due to less absorption by the muscle. Reasons for quadriceps weakness may include atrophy, reduction in the muscle fibers number, and changes in the muscle activation. People who had both asymptomatic patella-femoral and tibia-femoral radiographic knee OA have showed weaker quadriceps strength compared with healthy ones[77,78,79]. Reduced muscle strength is also reported to be associated with aging and augmentation of intermuscular adipose tissue which is clearly influenced by body weight[80]. Furthermore, a recent literature review concluded that muscle weakness may predict the onset and/or the progression of knee OA[81]. In addition, even though quadriceps strength enhanced under the setting of misalignment and laxity condition, the risk of tibia-femoral OA progression still increased[82]. However, another study denied this association, which makes us confused and suggests the presence of a complex concomitant interrelationship[83].

1.5.2.2 Repetitive joint use

Another biomechanical factor associated with an increased risk of osteoarthritis is repetitive joint use. In people whose jobs required occupational activities such as carrying and kneeling, the risk of development of knee OA was higher than two-fold compared to workers with other occupations, as confirmed by a recent meta-analysis[84-87]. Moreover, when overweight people take a job needing a physical activity, the risk of knee OA will be much higher[86,87]. Besides the job related occupational activities, some sports may be associated with increased risk of OA. In one study of athletes, knee injury among soccer players and increased BMI as well as squatting among weight-lifters seem to be related to higher risk of OA[88].

1.5.2.3 Joint injury earlier in life

A variety of studies have shown that knee injury is one of the strongest risk factors for OA. In the context of OA, the most important injury should be the rupture of anterior cruciate ligament (ACL), which is usually accompanied with damage of adjacent joint

structures[89]. In recent follow-up studies, those who experienced an ACL rupture or meniscal tear are associated with a higher risk of developing knee OA compared to general population[90-92]. Furthermore, direct damages to articular cartilage from instability after ACL rupture, or absence of load and friction dispersion from meniscal tear result in matrix disruption, chondrocyte necrosis and proteoglycan loss which maybe not reversible[93]. Though meniscectomy in treating meniscal tears helps people relieve the pain temporarily, they were more vulnerable to develop OA than others during the life course[94]. It seems that instead of being a risk factor, joint injury may rather act as an early sign of susceptibility to OA.

1.5.2.4 Joint misalignment

It is well known that anatomic angle and physical line were characteristics in normal femoral and knee joint. Misalignment may contribute to abnormal mechanical forces, as any shift from neutral or collinear alignment of the hip, knee and ankle affects load distribution at the knee. More recent findings reported that varus misalignment assessed by full-limb radiographs increased the incidence of both knee OA and cartilage damage[95,96]. However, the association between incident knee OA and misalignment is not so apparent, as others like Framingham study denies the relationship described above[97]. Furthermore, knee misalignment was considered to predict the progressive knee OA, but whether its correction will slow the disease progression or not, still needs to be figured out.

Risk factors of OA have been demonstrated by a vast of researches, but conflicts about the role of a single factor in developing OA still exist. Moreover, many other risk factors of OA are yet still unknown. The interplay relationship among these factors in contribution to OA is more complex than what we think about, which deserves to further researches.

1.6 Physiology of joint tissues and OA pathology

The normal articular joint is composed of synovial membrane, articular cartilage and subchondral bone. They construct a joint cavity filled with synovial fluid. Normal joint function is maintained by the interaction of joint components. Each of them having experienced alterations will render the whole joint, suffering from changes in structure

and function. If OA is considered as a communication among all of the alterations of joint components, it is necessary to understand the property of every component in physiological and pathological conditions to elucidate the disease mechanism (Figure 1.1.8).

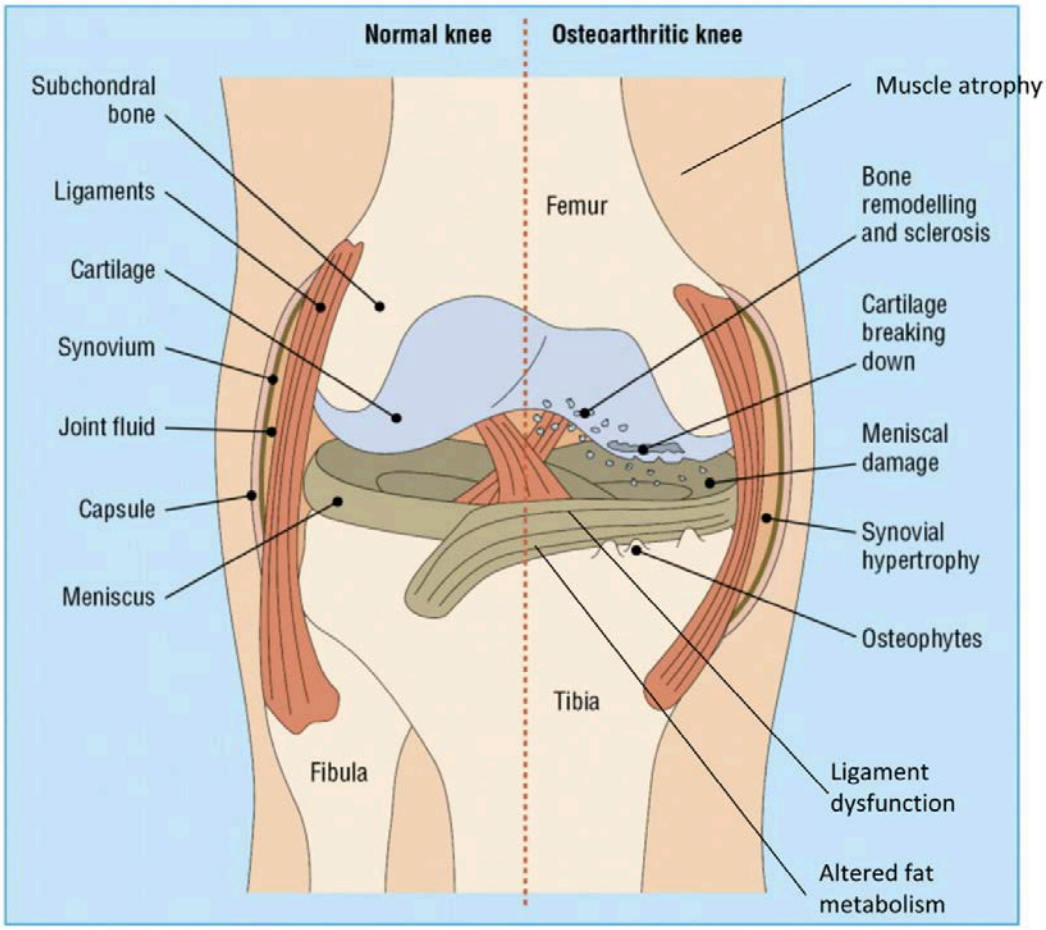

Figure 1.1. 8: The normal and OA knee joint. (DJ Hunter. 2011)

1.6.1 Synovial membrane

Synovial membrane, also known as synovium or stratum synoviale, is an important component of the synovial joint (Figure 1.1.9). The term synovium refers to the soft tissue lining the space of diarthrodial joints, tendon sheaths and bursae[98]. The synovium is a specialized connective tissue, subdivided for the purpose of analysis into cellular, ground substance and fibrillar components[99].

Synovium is very variable but often composed by two layers, intima and subintima. Cells in synovium are mainly macrophages and fibroblasts. The macrophages are

responsible for the removal of undesirable substances from the synovial fluid, while fibroblasts are in charge of hyaluronan (hyaluronic acid) manufacture, which is also the main structure of glycosaminoglycan in the cartilage. Together with lubricin, they render the synovial fluid more viscous and lubricant.

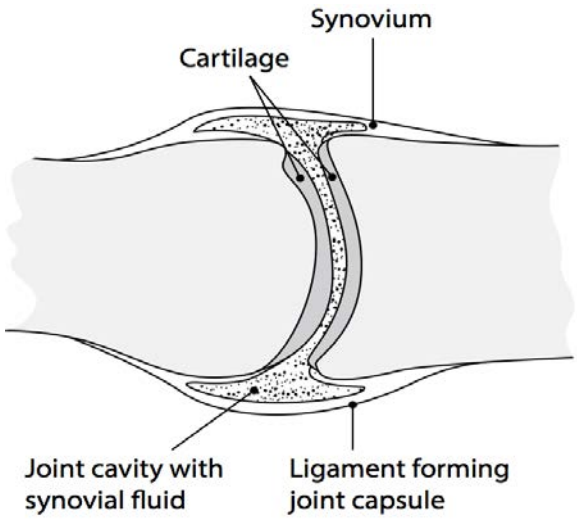

Figure 1.1. 9: Normal, healthy synovial joint. (Tamer T.M 2013)

1.6.1.1 Synovium structure

The outlayer, or subintima, forms the basis of the normal synovium, which can be divided into three subtypes : fibrious, areolar, and adipose[100]. Of these, the areolar form of synovium is the most specialized. As the underlying subintima is loose, the intima sits on a pliable membrane to form the synovial membrane. The subintima is composed of blood and lymphatic vessels and a cellular content of both resident fibroblasts and infiltrating cells in a collagenous extracellular matrix. Similar as in intestine, the subintima layer can form a microstructure called villi, which is considered to permit a much more contact surface with synovial fluid. Particularly, in addition to the similar function as that in intestine, this microstructure helps to change the shape during the joint movements, because it is commonly crimped into folds that may disappear when stretched. Based on this structure, a nutrition and waste exchange channel is constructed not only for synovium but also for articular cartilage.

1.6.1.2 Synovial macrophages and synovial fibroblasts

Cells in synovium are classified into two types, type A and type B. The former comprises macrophages while the latter includes fibroblasts. In the mature joint, the synovial lining is predominately occupied by macrophages, representing 10-20% of the cells, in addition to fibroblasts, and some cells with intermediate morphology[101].

Macrophages are found both in the intimal and subintimal regions of normal synovium, but differ in one to another. They are more numerous, have a prominent Golgi apparatus, numerous vacuoles, many filopodia, mitochondria, intracellular fibrils, micropinocytic like vesicles and manifest surface markers of the macrophage lineage[102]. The intima macrophages express the immunoglobulin receptor FcgRIIIa which is less present or absent in subintima macrophages, show a prominent nonspecific esterase (NSE) activity and are strongly positive for CD163 and CD68 but less for CD14[103]. Some evidences recently supported that macrophages in two layers, derived from bone marrow via circulating monocytes, and probably via subintimal venules[98,102,104]. Z39Ig, also expressed by synovial macrophages, is a cell surface receptor linked to the classic complement pathway. Expression of this receptor during macrophage differentiation induces the production of the matrix degrading enzyme MMP-9[105-108].

Synoviocytes B or fibroblasts are responsible for the synthesis of hyaluronan and its secretion into synovial fluid. The current view supports that the intimal fibroblasts have a single origin lineage able to give their special phenotype in response to local stimuli[109]. UDP Glucose dehydrogenase (UDPGD) is an enzyme that converts UDP-glucose into UDP-glucuronate, a vital substrate required by hyaluronan synthase for the production of hyaluronan[110]. The activity of this enzyme is a specific marker of synovial fibroblasts, reduction of which suggesting a disease state. The expression of CD55 is a specific marker for discriminating synovial fibroblasts from synovial macrophages[111-113]. In addition to lubricin, synovial fibroblasts synthesize several normal matrix components including laminin, collagens, fibronectin, proteoglycans. When exposed in a disease state, the synovial fibroblasts will be activated to produce cytokines, adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 and metalloproteinase[104].

1.6.1.3 Synovium Function

The functions of synovial tissue mean a lot to the joint, it is very difficult to define them exactly. The synovium maintains an intact non-adherent tissue surface, which allowed a continuous movement of the joint. Cells in synovial tissue are the major source of synovial fluid components, such as lubricin and hyaluronic acid. These components contribute to the maintenance of cartilage integrity by lubricating the cartilage surface as well as modulating chondrocyte metabolism. Moreover, synovial membrane provides nutrients essential for cartilage and removes products of chondrocyte metabolism and articular matrix turnover[114].

1.6.1.3.1 Maintenance of tissue surface

How joint surface becomes non-adherent to permit the joint movement? This maybe accounts by the hyaluronan in synovial fluid. Furthermore, plasminogen activator and decay-accelerating factor (DAF) from intimal fibroblasts may inhibit fibrin formation and scarring. In normal synovial joint, high molecular weight molecules like lubricin and hyaluronic acid (HA) are not readily permeable, while small molecules like growth factors and cytokines readily diffuse through the synovial membrane. On the other side, these high molecular weight molecules may keep a much higher osmotic pressure, which prevents the high molecular weight plasma membrane proteins from entering the synovial fluid[115]. If happened, the exotic proteins will not only alter the synovial fluid composition, but also settle on articular cartilage and induce a harmful antibody-antigen reaction. These functions are probably benefited by the intimal macrophages and fibroblasts. Enrichment of micro-vascular network beneath the synovial surface provides the nutrition and recruitment of new cells[98].

1.6.1.3.2 Lubrication

Lubricin, firstly named “lubricating glycoprotein 1” by David Swann, mainly secreted by synovial fibroblasts and to a lesser extent by chondrocytes, lubricates the joint[116]. Other molecules in the synovial fluid contribute to the low-friction and low-wear properties. These molecules act alone or in combination, such as proteoglycan 4 at 0.05–0.35 mg/ml, surface-active phospholipids at 0.1 mg/ml and HA at 1–4 mg/ml[117]. In addition to its weak lubricating power, HA seems to be more effective in maintaining synovial fluid constant during exercise.

1.6.1.3.3 Chondrocyte nutrition

Articular cartilage has no intrinsic vasculature or lymphatic supply, therefore the nutrition of chondrocytes and removal of metabolism products are linked to the synovial tissue and subchondral bone. Though blood vessels in the synovial membrane were suggested to be vital for cartilage nutrition, no special structure has been found to account for this function. Because blood flow in subchondral bone is three to ten times higher than in trabecular bone, the blood flow to subchondral bone supplies approximately 50% of the glucose, oxygen, and water required by articular cartilage[118,119].

1.6.2 Pathological synovium

OA was categorized as a non-inflammatory form of arthritis for a long time, but this condition has been changed. Houli *et al*. were among the first to describe features of inflammation in synovial tissue from OA patients[120], and a variety of researches confirmed the presence of inflammation[115,121-123]. The synovial inflammation or synovitis in OA is represented by infiltration of mononuclear cells, though this inflammation is less severe compared with RA, it is always higher than that in healthy controls[115]. A frequent used scoring system for synovitis, which is based on morphologic alterations such as hyperplasia/enlargement of synovial lining cells, activation of resident cells/synovial stroma and inflammatory infiltration, has been developed by Krenn *et al*.[124]. With this scoring system, a range for the severity of synovitis has been reported. In presence of a scale 0 to 9, mean score in OA patients is around 2, while 1.4 for healthy controls and 5.7 for RA[121,125-127].

1.6.2.1 Cellular aspect of synovitis

Inflammation of the synovium has been seen primarily as a lymphocytic phenomenon. Though the accumulation of lymphocyte aggregates and germinal centers in the synovium was observed, these changes palely account for the arthritis pathology. In synovitis, the main immune cells found were T cells, macrophages and mast cells, but neutrophils were almost never found[122]. T cells were predominantly detected in the sublining layer and to some extent in the deep layer, represented by $CD4^+$ and $CD8^+$ cells[128]. After exposed to auto-antigens released from cartilage such as chitinase-3-like

protein 2 (YKL-39) and type II collagen peptides, T cells can be activated in situ leading to an angiogenic organization[129]. Based on the cytokine profile upon *in vitro* activation, several types of T helper (Th) cells have been identified in OA synovium, such as Th1, Th2, Th17 and regulatory cells (Th3 and Tr1)[130-133]. Among them, much more Tr1 cells are present in OA synovial tissue than that in RA, which indicates its inhibitory role in immune responses[134]. Macrophages are mostly distributed in the lining layer. Though emphasized, their role in contribution of OA synovitis is yet unknown. Compared with T cells and macrophages, mast cells and B cells are less present. The highest number of mast cells was present in superficial layers of OA synovial tissue, while in the capsule for RA[135]. B cells have been also detected in the infiltrate in half of OA patients. They were shown to be oligoclonal and this antigen-driven expansion may contribute to de development or progression of OA[136,137].

1.6.2.2 Vascular aspect of synovitis

The vascular aspect of synovial inflammation is crucial, as increased angiognesis has been reported in synovial tissue, cartilage and menisci. In the synovium, the endothelial cells can be activated by several angiogenic factors, following by release of proteolytic enzymes which are responsible for the degradation of the basic endothelial membrane. Thereafter, the activated endothelial cells help to synthesize a new basement membrane. Angiogenesis, which forms an integrated process with inflammation and may affect disease progression and pain, is associated with chronic synovitis but occurs at all stages of OA[138,139]. On the other side, the transport of inflammatory cells and mediators could perpetuate the angiogenesis, which forms a feed-back control[140]. Angiogenesis in OA synovitis has been examined with several technologies such as histology, synovial cell cultures, animal models and Doppler ultrasonography[114]. For instance histological studies demonstrated an increase in the vessel size and vascular density. In addition, OA synovial cell cultures strongly expressed vascular endothelial growth factor (VEGF) levels. Intra-articular over-expression of anti-angiogenic factors suppresses synovium inflammation, osteophytes formation and cartilage degradation in animal models.

1.6.2.3 Inflammatory mediators in synovitis

The involvement of inflammatory cytokines, chemokines and other inflammatory mediators may account for the synovitis in OA, their production were induced by the activation of specific transcription factors, with NF-κB playing a prominent role. In addition, the molecular products derived from cellular stress and extracellular matrix disruption, innate immunity, neuropeptides and mediator of pain could also contribute to the OA synovitis progression. A schema was designed to interpret the relationship between inflammation, angiogenesis and cartilage degradation in OA (Figure 1.1.10).

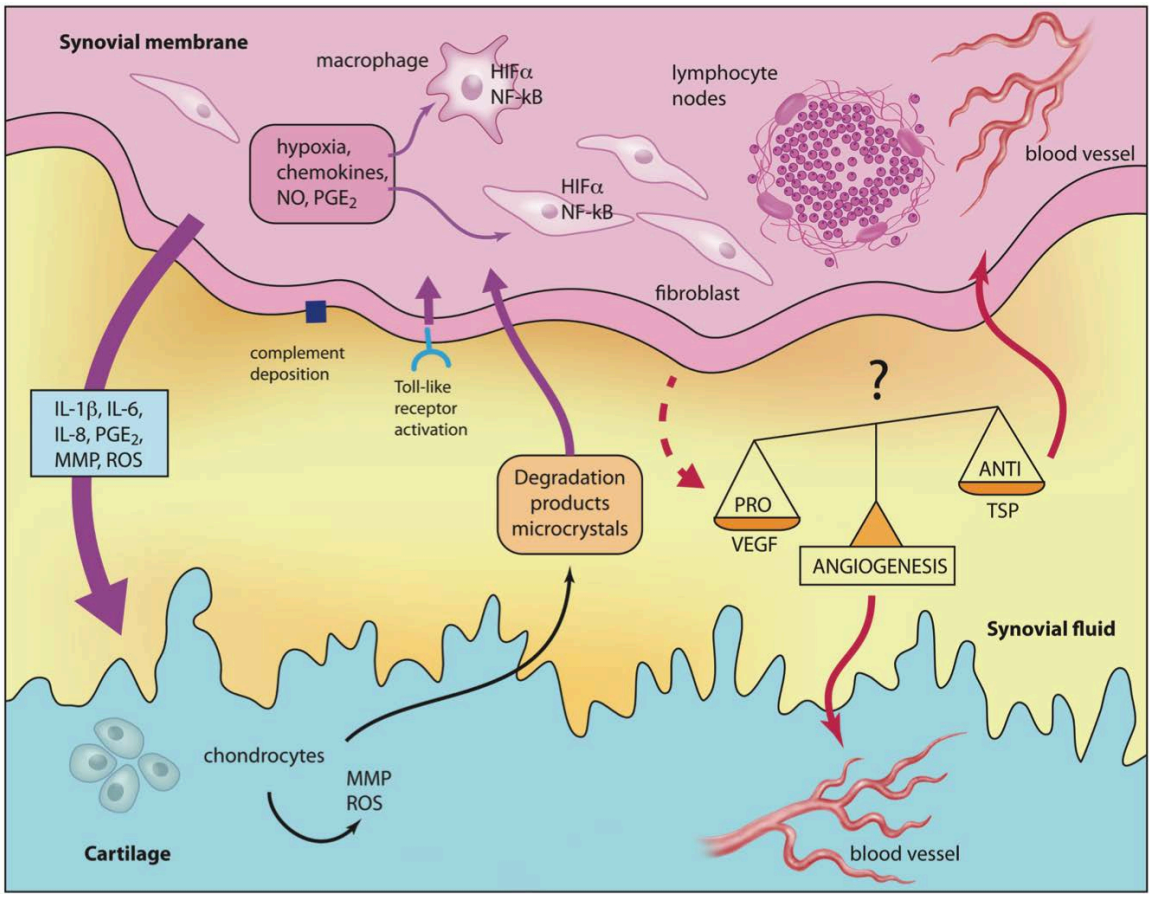

Figure 1.1. 10: Schematic representation of relationship between inflammation, angiogenesis and cartilage degradation in OA. HIF, hypoxia-induced factor; IL, interleukin; MMP, matrix metalloprotease; NF-κB, nuclear factor-κB; NO, nitric oxide; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; TSP, thrombospondin (Herontin Y *et al.* 2014).

Interleukin (IL)-1β and TNF-α are the major inflammatory cytokines released into the synovial fluid during the synovitis. Though their cellular sources are still largely

unclear, it seems that macrophages play an important role. Via combined effects of IL-1β and TNF-α, macrophages regulate possibly the expression of other cytokines such as IL-6 and IL-8, and promote cartilage catabolism by producing matrix-degrading enzymes in both synoviocytes and chondrocytes[141]. In addition, compared to late OA patients, more IL-1β and TNF-α have been found in early OA patients, who are characterized by knee pain, normal radiographs but arthroscopic signs of OA[142]. In view of these data, these cytokines were largely investigated and presented as prominent cytokines in the OA pathogenesis. Attempts to therapeutically block their activities in patients have been conducted. Although the trials did not make their words, the improvement of pain and physical function scores was reported in some individuals[143,144].

In the last decade, Gal-3 has been discovered to be involved in the initiation/progression of synovitis. This is a soluble animal lectin of 30 kDa that preferentially recognizes lactosamine and N-acetyl-lactosamine structures. Frequently investigated in cancer pathology, Gal-3 was also found at a high concentration in synovial tissues and serum from RA patients. Though less Gal-3 was found in OA, but still higher than in healthy control[145], it has been reported that Gal-3 participates in a variety of processes[146]. During OA, activated synovial macrophages secrete Gal-3 into the synovial fluid, and this localization has been shown to impair joint tissues. MMP-3 and A Disintegrin And Metalloproteinase with Thrombospondin Motifs (ADAMTS) 5, enzymes involved in cartilage degradation, were stimulated by Gal-3 in the chondrocytes[147].

Furthermore, except for the cytokines described above, several other pro- and anti-inflammatory cytokines in OA synovium have been discovered as well, such as IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10 IL-15, IL-18, IL-21, TGF-β[122]. However, cellular sources have not been identified for most of them and more researches deserve to figure that out.

The involvement of innate immunity has been described in synovitis, including the recognition of distinct pathogen-associated molecular patterns (PAMPs), damage associated molecular patterns (DAMPs) and complements by pattern recognition receptors (PPRs). Expressed by macrophages and other synovial lining cells, these PPRs will be activated to induce the production of pro-inflammatory cytokines and chemokines leading to the inflammatory response.

Toll-like receptors (TLRs), the type I transmembrane glycoproteins, are members of the largest PRRs[148,149]. TLR-2 and TLR-4 are involved in the activation of the synovium and inflammation, and their stimulation was shown to induce IL-15 production in vitro[150]. IL-15 was consistently detectable and elevated in patients with early stage of OA, which indicates indirectly the role of TLR-2 and TLR-4 in the OA progression[151]. The receptor for advanced glycation end-products (RAGE) is also a member of the PRR family, and its up-regulation has been associated with the inflammatory process of OA[152]. Endogenous molecular products, such as microcrystals, complement components, matrix fragments and products of cell death and matrix catabolism can function as DAMPs to activate TLRs and RAGE. Interestingly, as TLRs and RAGE are all glycoproteins, Gal-3 virtually should be put on a more prominent position due to its special affinities towards β-galactoside structures.

There are more than 30 plasma and membrane-bound proteins which have been discovered in the complement system, they can be activated in three pathways: the classical, the alternative, and the lectin pathways[153]. The main function of complement is to help the immune system to eliminate "foreign" particles and macromolecules which are present in our body. The components of complement have been detected in OA synovial fluid, and their deposition in the synovium of patients with cartilage degradation is observed[154,155]. The C3a, C3b and C5 fractions can be activated by fibromodulin, COMP, and osteoadherin, which results in the promotion of inflammation and phagocytosis[123].

There are other contributions to synovium pathology. Hypoxia is believed to be responsible for the synovitis by stimulating inflammatory cytokines, represented by induction of gene expression involved in angiogenesis, inflammation and tissue degradation. HIF-1 is considered to carry out the hypoxic effect directly, particularly its subunit HIF-1α.

The production of ROS and MMPs by synoviocytes due to the NF-κB activation, was showed to be involved in the damage of local tissue as well as in recruitment and activation of the immune cells[114]. Furthermore, substance P is a neuropeptide found in the subintimal portion of the OA synovial membrane, close to osteophyte or cartilage lesion. It stimulates synoviocytes proliferation and their production of prostaglandin E2 (PGE_2) and collagenase[156].

1.6.3 Cartilage physiology

The cartilage plays an important role in the joint movement. Though very thin, it provides a special buffer cushion for the joint, by distributing loads and minimizing peak stress on subchondral bone. The chondrocytes in the cartilage also share a role with the synovial cells in maintaining lubricants (lubricin and HA) level on the articular surface, which permits pain-free movement with a low level of friction[157]. However, cartilage is not very powerful to maintain and repair itself due to its avascular and aneural structures, and this poor ability of repair will progressively decline with aging.

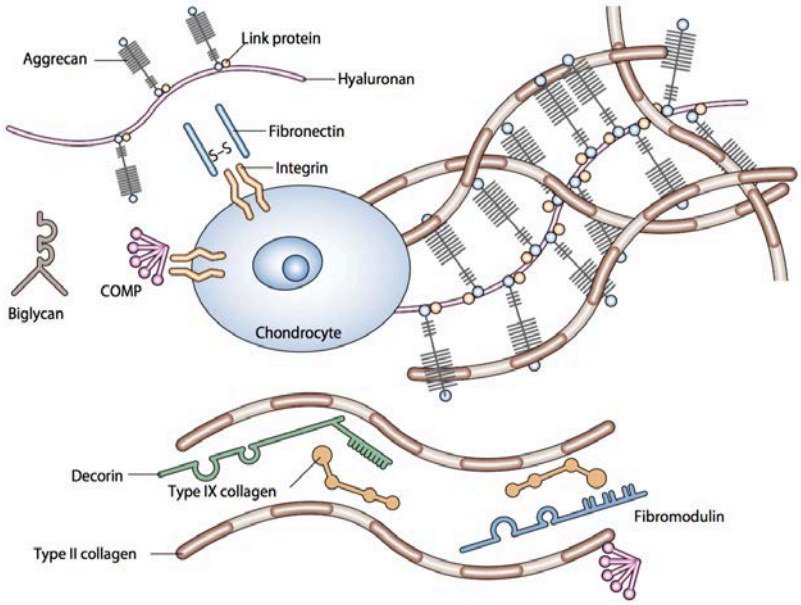

Figure 1.1. 11: Components and structure of normal cartilage. (Tamer TM *et al*. 2013)

1.6.3.1 Cartilage components

The articular cartilage is composed of an extracellular matrix filled with sparse population of chondrocytes. The extracellular matrix consists of tissue fluid and the framework of structural macromolecules. Water in the tissue fluid contributes up to 80% of the weight of the articular cartilage, while collagens, proteoglycans and other non-collagenous proteins account for the dry weight of the cartilage. Chondrocytes contribute to approximately 10% of the weight of articular cartilage, their presence allow the maintenance of the extracellular matrix[158-160]. A schema designed by Tamer TM *et al*. described the cartilage components and structure (Figure 1.1.11).

1.6.3.1.1 Collagens

Regardless of the predominant collagen in the cartilage, which is type II, several other types of collagen have also been identified, such as types I, III, V, VI, IX, X and XI[161-165] (Table 1.1.3). Formed by three covalent cross-linked polypeptide chains, the collagens intertwined with proteoglycan aggregates in the extracellular matrix. This structure does not offer resistance to compression, but endows the cartilage with a tensile property[158].

Table 1.1. 3 Different cartilage collagens and their functions. (Bhosale AM *et al*. 2008)

Collagen type	Morphological location	Function
II	Principal component of macrofibril (90–95%)	Tensile strength
VI	Pericellular matrix	Helps chondrocytes to attach to the matrix
IX	Cross-linked to surface of macrofibril	Tensile properties and inter-fibrillar connections
X	Closely related to the hypertrophied cells in calcified cartilage layer	Structural support and aids in cartilage mineralization
XI	Within or on macrofibrils	Nucleates fibril formation

1.6.3.1.2 Proteoglycans

Proteoglycans are produced inside the chondrocytes and secreted into the matrix. They represent the second-largest group of marcromolecules in the extracellular matrix of the cartilage. Proteoglycans consist of a protein core, to which glycosaminoglycans (chondroitin sulfate and keratan sulfate) are attached to form a bottlebrush-like structure[166]. These proteoglycans, such as decorin, biglycan and fibromodulin, are characterized by their abilities to interact with collagen[167]. Furthermore, some proteoglycans aggregate with HA via link proteins to form large proteoglycan aggregates, called aggrecans[159]. Aggrecan contributes to about 90% of the proteoglycan mass in the cartilage and helps to prevent a complete displacement of water during the tissue deformation.

1.6.3.1.3 Chondroytes

Chondrocytes are the specialized cells in the cartilage, they orchestrate the balance between matrix synthesis and breakdown to maintain normal tissue metabolism[168]. Due

to their sparse distribution in the cartilage, no cell-to-cell contacts for signal transduction and communication between cells are found. Chondrocytes vary in shape, number and size depending on the located regions. They receive nutrition from the adjacent tissue and synthesize the collagens and proteoglycans to maintain the extracellular matrix. Various collagens are expressed in chondrocytes at different stages. Chondroprogenitor cells are characterized by the expression of type II collagen, type IIA procollagen (COL2A), mature chondrocytes express the typical cartilage collagen types II (COL2B), IX, and XI as well as aggrecan and link protein, while the hypertrophic chondrocytes express type X collagen[169]. Since the cartilage is avascular, the chondrocytes must get nutrition by facilitate glucose transport. This facilitation is conducted by the constitutive glucose transporter proteins, GLUT3 and GLUT8[170] and production of a HIF-1α. In addition, up-regulation of HIF-1α has been found in chondrocytes when cultured at low oxygen tension in vitro[171].

1.6.3.2 Cartilage layers

The ECM around chondrocytes is divided into pericellular, territorial and inter-territorial matrices. Chondroitin sulphate rich proteoglycans and keratin sulphate rich proteoglycans are the main proteoglycans of these three territorial matrices respectively, in which the collagen fibers ranged from the thinnest to the largest[160,172]. With the help of microscopy, a horizontal matrix subdivision of the cartilage has been established: the superficial zone, the middle zone, the deep zone, and the calcified zone[173-175] (Figure 1.1.12). Various numbers and shape of chondrocytes, different concentration of extracellular matrix components are detected in the four zones described above, which suggests a different role for each zone.

1.6.3.2.1 Superficial zone

Chondrocytes in this zone are mostly flat, with the highest cell density of cartilage, which may reflect their adaptation to the joint movement. They synthesize a matrix containing higher collagen concentration and lower proteoglycan concentration compared with other cartilage zones. The collagen fibrils are arranged parallel to the surface. This structure provides to the superficial zone, tensile stiffness and strength, which may resists to shearing forces generated during joint use. This zone also serves

as a barrier to prevent the movement of large macromolecules, which protect the cartilage from the synovial tissue immune system.

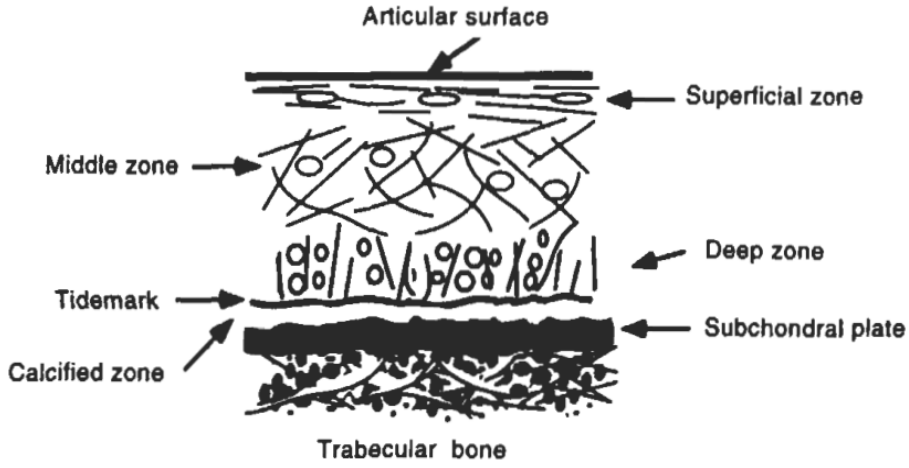

Figure 1.1. 12: The cross-sectional view through the articular cartilage into the subchondral bone. The circular objects are chondrocytes. The shape of chondrocytes and orientations of collagen fibers differ in different layers. (Cohen NP *et al*. 1998)

1.6.3.2.2 Middle zone

As the name indicates, the morphology of chondrocytes and matrix composition are intermediate between the superficial zone and the deep zone. Compared with superficial layer, less numerous chondrocytes are present in this zone, and cell shape turned into spheroid. They synthesize higher concentration of proteoglycans and larger diameter collagen fibers which are not well organized but are typically in an oblique orientation to the surface.

1.6.3.2.3 Deep zone

Compared with other layers, the largest diameter of collagen fibers associated with the lowest number of chondrocytes are oriented in vertical perpendicular to the joint surface. The highest concentration of proteoglycans and the lowest concentration of water are found as well. The tidemark, where the collagen fibrils end, is a thin basophilic line seen on light microscopic sections separate the non-calcified cartilage to the calcified cartilage.

1.6.3.2.4 Calcified zone

Cells in this zone show low metabolic activity, with a hypertrophic phenotype. Unlike chondrocytes of other zones, type X collagen was synthesized by chondrocytes in calcified zone suggesting its role in cartilage mineralization. The calcified cartilage is the boundary between the cartilage and the underlying subchondral bone, which provides a buffer area.

1.6.3.3 Articular Cartilage properties

The articular cartilage is characterized by providing a low-friction gliding surface and act as a shock absorber that minimizes peak pressure on the subchondral bone. The interaction meshwork constituted by chondrocytes and their extracellular matrix may account for normal cartilage functions.

The biphasic nature of the cartilage was considered to be a prominent characteristic in response to the load exerted on the joint. The fluid phase is characterized mostly by water, combined with inorganic ions such as sodium, calcium, chloride, and potassium. The solid phase is represented by the extracellular matrix, the network formed mainly by collagen and aggrecans, which is porous and permeable[158,159].

When the pressure changes on the articular cartilage, the fluid phase will flow through solid phase (ECM). This fluid flow, which exerts a large frictional drag on the solid matrix, could interpret the viscoelasticity and compressive properties of articular cartilage. The viscoelasticity behaviors are composed of two phenomena[176,177]. One occurs when a constant loading is exerted on the cartilage which will deform to adapt until the load is balanced. The other takes place when the deformed cartilage is held at a constant strength until the stress reaches zero where the equilibrium happens. As for the compressive behavior, the flow through the solid phase is believed to play a dominant role. Not only clinical observations but also *in vivo* animal studies have shown that joint loading can induce various cartilage metabolic responses. The dynamic loading induces the production of proteoglycans, while severe impact, strenuous exercise, acute and chronic injurious loading contribute to the cartilage degradation[178,179]. *In vitro*, Buckwalter J.A *et al*. demonstrated that static compression inhibits the matrix component synthesis, whereas the dynamic pressure stimulates the synthesis[178].

As described above, collagen fibers differ in different zones, not only their diameters but also the orientations. This special structure, particularly the orientation of the collagen network, leads to the inhomogeneity of the cartilage tensile properties. The split lines are lines of cleavage formed along the planes of weakness in the cartilage. Cartilage specimens that harvested parallel to the split lines are stiffer than those harvested perpendicular to the split lines. Although much less frequent, significant shear stresses are also present in the cartilage, particularly in the deep zone near the tidemark. In fact, the overall stiffness of articular cartilage in shear is much more related with the amount of collagen rather than the proteoglycans. However, proteoglycans help to hold the collagen network in place.

1.6.4 Cartilage pathology in OA

OA is considered to be a disease of the joint as an organ, but it affects predominantly articular cartilage characterized by the progressive loss of its normal structure and function. Although severe impact or strenuous exercise loading, acute or chronic injurious compressive overloads will result in cartilage degeneration during OA, ageing is considered a more common factor[180-183]. The earliest visible sign of OA is fibrillations of cartilage surface, which extend to the deeper zones of cartilage until the fissures reach subchondral bone[184]. Moreover, the subchondral bone remodeling will in turn favor the cartilage degeneration. With the disease progression, cartilage surface is torn and cartilage fragments are released into the joint space. These fragments, will activate TLRs and RAGE leading to the innate immune response by synoviocytes, which aggravates the cartilage degradation[185]. Though the chondrocytes attempt to repair the damage matrix by production of the corresponding components, finally they fail. Complete loss of cartilage results in the direct contact of bone-to-bone, which accelerates the OA process.

In the early stage of OA, the proteoglycan aggregation and aggrecan concentration decrease accompanied with induction of the water content, but the collagen framework remains stable. The abruption of proteoglycan-collagen interface will lead to the swelling of cartilage matrix, increases the permeability and reduces the stiffness of the tissue[186]. As the intact matrix network permits the normal mechanical behavior of cartilage, the disruption will influence its weight-bearing capacity. Once the tissue alterations are detected by chondrocytes, these specialized cells will attempt to reverse

their low metabolism state to compensate the damage. They not only increase the synthesis of matrix macromolecules to reconstitute the network, but also stimulate the production of cytokines and chemokine receptors. The matrix synthesis depends on the response to various anabolic cytokines and growth factors, such as TGF-β, bone morphogenetic proteins (BMPs), and IGF-1. It is reported that mature BMP-7 is down-regulated in OA cartilage while its inactive form remain high, suggesting the involvement of the conversion of inactive BMP-7 to an active form in matrix homeostasis[169].

Numerous studies show that TNF-α and IL-1, the main inflammatory cytokines detected in synovitis, can be produced by OA chondrocytes as well. These cytokines, along with cartilage degraded products such as fibronectin fragments, can stimulate collagenases, aggrecanases (ADAMTSs) and nitric oxide synthase expression[182]. MMP-3 and ADAMTS-5 are responsible for aggrecan degradation in early OA, while in late phase MMP-13 is highly efficient at degrading type II collagen which is usually not accessible due to the coating of proteoglycans[187]. Van der kraan PM *et al*. proposed a schema to describe the articular cartilage alterations during the OA progress (Figure 1.1.13). In addition to direct cartilage degradation, MMPs can also release several growth factors from the ECM or the cell surface, which could confer to the anabolic activity of chondrocytes[188]. In turn, the NO production by the chondrocytes can induce IL-1 production, which consequently promotes the expression of inflammatory cytokines[184]. The main source and disrupted origin of these proteolytic enzymes differ from RA to OA. The former takes inflammatory synovium as principle source with origin activity at the cartilage-pannus junction as well as in deeper zones of cartilage matrix, while the latter shed light on the stimulated chondrocytes partly combined with the synovium and the destruction arised from the cartilage surface[170,189].

In addition, the age related changes in the cartilage include the induction of ROS, which are susceptible to mediate cell death. This is interpreted by the dysfunction of mitochondria and results in DNA damage and telomere shortening, which can be promoted by inflammatory cytokines such as TNF-α and IL-1[183]. Cell death in OA mostly occurs in the cartilage calcified layer, death products such as pyrophosphate and precipitated calcium may contribute to pathologic cartilage degradation[169]. Once chondrocytes fail to stabilize or restore the tissue, progressive loss of articular cartilage takes place. DNA polymorphism and methylation are reported as well, and the latter

can be targeted by transcription factor inhibitors, cytokines, and drugs (such as BAY, an NF-κB inhibitor, and leptin) in *in vitro* experiments[190,191].

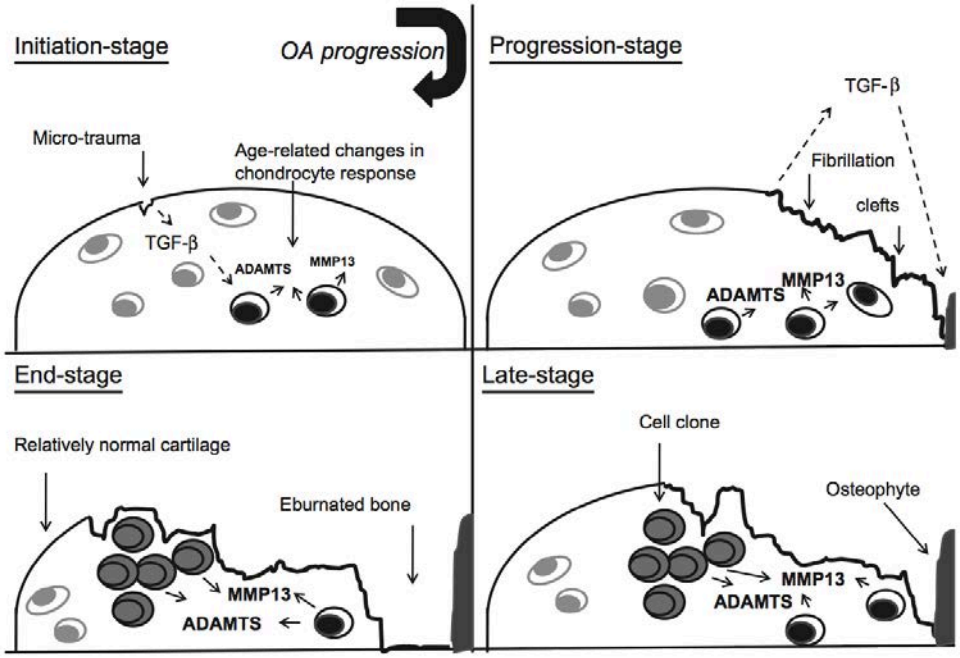

Figure 1.1. 13: Chondrocyte hypertrophy-like changes in a conceptual model of human primary OA. (Van der kraan PM *et al*. 2012)

1.6.5 Subchondral bone physiology

The subchondral bone (SCB) is also a prominent component of the joint. The bone underlying the cartilage includes SCB plate, trabecular bone and bone marrow space. However, most of the time, the SCB is believed to refer to the bony component just beneath the calcified cartilage, which means SCB plate. The line that separates the calcified cartilage from the SCB plate is called the cement line[192].

1.6.5.1 SCB ECM

ECM in SCB is mainly constituted by collagenous proteins, complemented by a few non-collagenous proteins. The former are represented mostly by type I collagen with subtle concentration of type III and type V collagen, while the latter are composed of growth factors and other molecules in trace amounts[193]. The collagens are oriented in a preferential direction giving to lamellar bone its structure, and hydroxyapatite

[$Ca_{10}(PO4)_6(OH)_2$] crystals are distributed on and around the collagen fibers to form the mineral content of the bone[194]. Non-collagenous proteins are present at the same molar basis as collagen, such as proteoglycans, glycosylated proteins with potential cell-attachment activities, and γ-carboxylated (gla) proteins[195]. Several growth factors have been found in non-collagenous proteins including TGF-β, IGF, BMPs, platelet-derived growth factor, and fibroblast growth factors (FGFs) that are supposed to be important for osteoblast stimuli during bone remodeling[196]. The main glycosylated protein present in bone is alkaline phosphatase (ALP), which plays a crucial role in the mineralization[197].

1.6.5.2 SCB cells

Three cell types were discovered in the SCB: osteoblasts, osteocytes and osteoclasts. Normally, the bidirectional interaction between osteoblasts and osteoclasts maintains a balance between bone formation and bone resorption (Figure 1.1.14). Thus, bone remodeling is in balance and the bone mass remains constant. Osteoblasts derive from mesenchymal cells and are characterized by forming bone. Various proteins are synthesized by osteoblasts, the most prominent is type I collagen fiber, with osteocalcin and bone sialoprotein recognized as specific proteins for bone[198]. Osteoblasts, once covered by their own secreted matrix, are called osteocytes. Though the extracellular matrix in the cortical bone (SCB plate) and trabecular bone is similar, it has been hypothesized that osteoblasts or osteocytes may behave differently according to their location[198]. This would suggest that osteoblasts respond differently to systemic hormones such as parathyroid hormone (PTH) and 1,25$(OH)_2$-vitamin D_3 in different parts of the skeleton[69,198,199]. Moreover, the receptors for these two bone resorbing hormones are presented in osteoblasts, which hints the role of osteoblast in the formation of osteoclast[200]. Osteocytes are presented as main cells in both cortical bone and trabecular bone and are responsible for the bearing of mechanical load, mechanoreceptors on the cell surface favoring this ability[201]. Furthermore, the osteocytes can express the components of the Wnt/β-catenin pathway which is a crucial signaling pathway in controlling the function of osteoblasts, and direct osteoclasts to the site of the damage, hastening healing[202-204]. The space occupied by each osteocyte and its matrix is known as a lacuna, unlike the chondrocytes, osteocytes can interact with each other. Osteoclasts are originated from the fusion of mononuclear progenitor cells, the differentiation of which requires the stimulation of the cytokine macrophage

colony-stimulating factor (M-CSF)[205]. Osteoclasts are the only cells in nature that can degrade mineralized bone tissue. Similarly as osteoblasts, they are different in various parts of the skeleton[206,207].

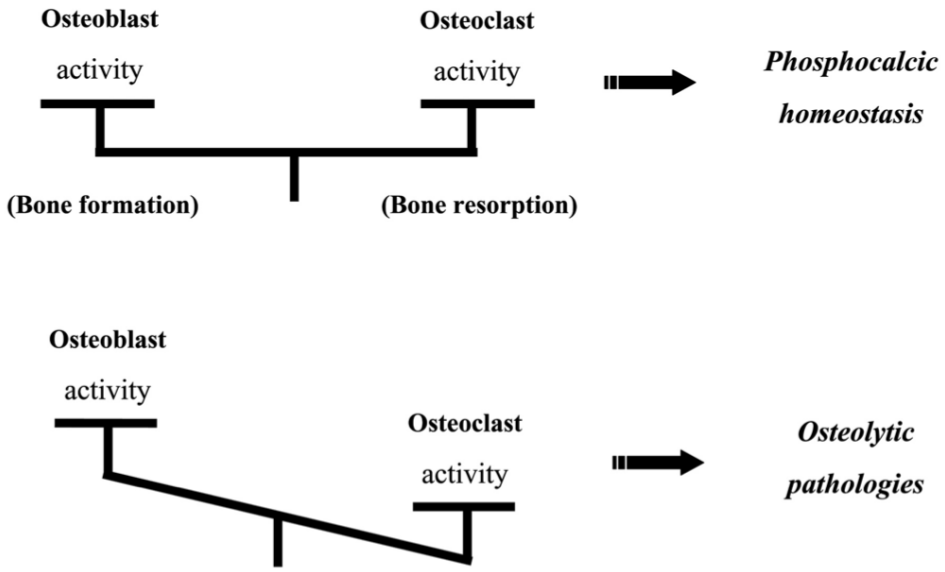

Figure 1.1. 14： The balance of bone formation and resorption in normal and pathological conditions. (Kwan Tat S *et al*. 2009)

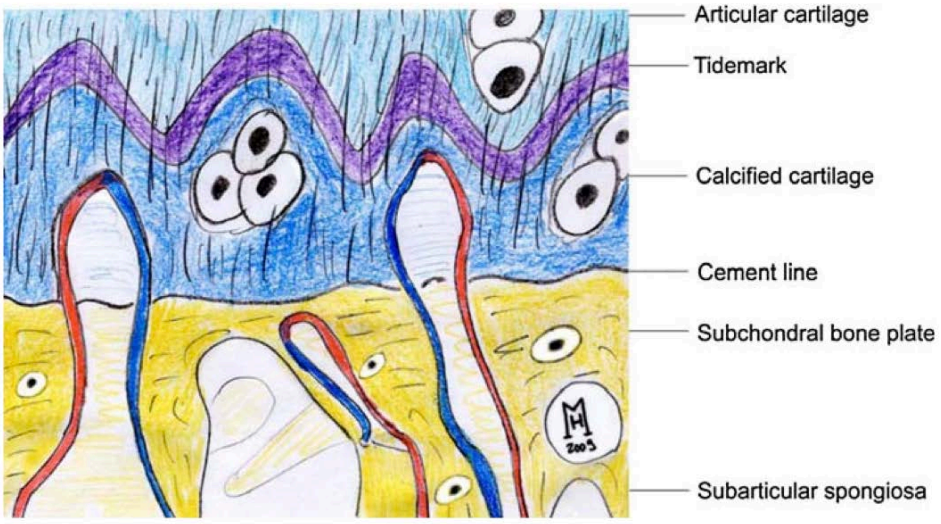

Figure 1.1. 15: Detailed schematic drawing of the calcified cartilage layer and the subchondral bone plate with articular vascular plexus vessels. (Mandry H. 2010)

1.6.5.3 SCB characteristics

A line of demarcation called the tidemark separates the calcified cartilage and non-calcified cartilage. The type II collagen fibrils go across the tidemark to reach the calcified cartilage, which constructs a continuous collagen network of the cartilage. However, the type II collagen fibrils do not extend to the SCB. Instead, the type I collagen served as main collagen in the SCB, which also make a continuous collagen network in trabecular bone[208]. The porosity of SCB plate renders itself permeable, represented by various channels favoring the direct communication. The shape and diameter of these channels are various according to the SCB changes. A number of blood vessels and nerves cross the channels and distribute tiny branches into the calcified cartilage[192,209] (Figure 1.1.15). Osteochondral junction, an integrated complex between cartilage and SCB, is defined to be composed of deep layer of non-calcified cartilage, the tidemark, calcified cartilage, the cement line and SCB[210,211]. This complex not only contributes to normal joint function, but also jeopardizes it in the disease such as OA[210].

1.6.5.4 SCB physical function

The SCB plate provides support for the overlying cartilage, absorbs the mechanical force exerted on the joint. Although we have described the bearing-load property of the cartilage before, SCB attenuates the loads at about 10 times than cartilage[212]. The architecture of SCB adapts to changes of the mechanical stress transmitted by diarthrodial joints. The thickness of the SCB plate also varies within the joints. For instance, the center of the tibia plateau is thicker than at the periphery, the difference can reach 7 to 12 times. The elevated thickness in the center is associated to another physiologic adaptation of bone. Some holes are distributed in greater stress regions accompanied with an increase of vessel density, thus favoring a better nutritious and oxygenation status[213-215]. Similar findings are reported in patella and ulna[215-219]. In addition to the vascularity and thickness, more abundant mineralization and more strength of the SCB plate are found in the heavily loaded regions[220-224]. An incongruent joint surface is maintained by SCB, the outer surfaces are predominantly in contact while center surfaces remain separated[225] (Figure 1.1.16). This shape permits the axial move of the center joint and transmits the stress to the cortical bone during loading, without influence on the nourishment of the overlying cartilage[226].

1.6.6 SCB pathology in OA

Though OA has been described as a primary disorder of articular cartilage, accompanied with moderate inflammation of the synovial membrane, the contribution of SCB should not be ignored. The origin of OA is still debating, particularly between the bone and cartilage. Physiologically, the osteochondral junction is in favoring the function interaction. In OA, the cartilage degradation contributes to SCB deterioration, in turn, once SCB damage occurs, the overlying cartilage may suffer by a secondary destruction. With this vicious circle, OA is initiated and progressed.

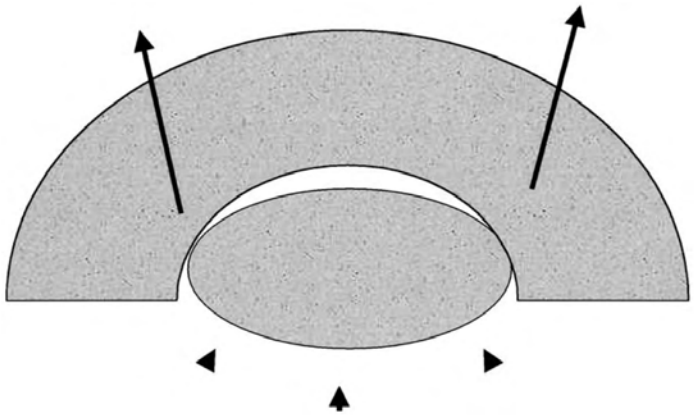

Figure 1.1. 16: A schematic diagram demonstrating an incongruent joint surface with the imposed load (arrowheads) leading to subchondral bone bending and transfer of the load to the cortical bone (arrows). (Kawcak, C. E *et al*. 2001)

1.6.6.1 SCB degradation characteristics

During the OA process, the alterations of SCB are characterized by progressive increased bone thickness termed bone sclerosis, formation of new bone at the joint margins termed osteophyte, the development of SCB cysts and MRI-visualized bone marrow edema-like lesions (BMELs). A model for the generation of SCB alterations in OA has been described by P Orth *et al*. (Figure 1.1.17). However, regardless of the bone sclerosis in the late stage of OA, a paradox is hypo-mineralization, which suggests an abnormal bone turnover[227]. The upward SCB plate and the advancement of the tidemark which are considered of the results of the vascular invasion from SCB into the calcified cartilage are also important alterations during the OA progress[228]. All of the

SCB alterations have been confirmed by accumulating evidences, not only in preclinical animal models, but also in specimens retrieved from OA patients[228,229].

1.6.6.1.1 Bone sclerosis

In fact, although bone sclerosis is considered as a hall mark of OA, SCB loss has been discovered in the early stage of OA in human, reflected by a thinner and more porous structure with high incidence of micro-damage[230,231]. SCB alterations have been confirmed accompanied with thickness reduction of the underlying trabecular bone and induction of osteoclasts in animal models[232-236]. Though the mechanism of elevated bone remodeling in the early phase of OA is still obscure, micro-damage repair, increased vascularity by angiogenic factors and promoted bone-cartilage interplay are proposed to be involved[211,237-239]. As SCB is in charge of the load attenuation in joint, its alterations during OA transmit higher loads to the overlying cartilage, yet the aggravating of cartilage destruction. In the late stage of OA, SCB turns to be sclerotic which is represented by the increase of osteoid matrix deposition meanwhile an abnormal mineralization has been identified[229,240-242]. The SCB density and volume increase corresponding to elevated trabecular bone thickness, decreased trabecular separation and bone marrow spacing[240,243]. Growth factors such as IGF-1 and TGF-β, which can stimulate osteoblast anabolic activity, accumulate in sclerotic SCB[241,243]. Moreover, OA osteoblasts produce a variety of homotrimeric alpha1 form of type I collagen, which has less affinity for the calcium than the normal (α1)2 α2 type I collagen form of collagen, and therefore is responsible for the low mineralization of the collagen matrix of SCB[242]. All of these events can account for the abnormal bone remodeling in late stage of OA and suggest that the abnormal osteoblast phenotype plays a crucial role in the disease progression rather than just contributing to bone sclerosis.

1.6.6.1.2 Osteophytes

Usually, osteophytes, seen radiologically, are found at the margin of the joint. Patients will be diagnosed as OA due to the osteophyte formation associated with pain and function loss. However osteophytes can also be present without symptoms which lead to few attention paid by potential OA patients[244]. In human, osteophytes formed via the endochondral bone formation are well accepted[245-247] whereas their formation is intramembranous in animal models[240]. During OA, bone marrow derived mesenchymal

stem cells (MSC) and/or chondrocytes undergo hypertrophy, followed by endochondral ossification, bone deposition and formation of marrow cavities at the joint margin[244,248]. Growth factors such as TGF-β and BMP-2 are implicated in the early and later process respectively, which are confirmed by experimental approaches[244,249-251]. Moreover, when transcription factor Runx2 required for chondrocyte hypertrophy is knocked out in mice, the OA development after joint injury is countered[252]. Whether osteophyte formation is a functional adaptation or only a pathological phenomenon in OA is unclear, but there is no doubt that the endochondral ossification indeed plays an important role in cartilage disappearance and bone remodeling.

1.6.6.1.3 Bone marrow edema-like lesions

The presence of BMELs in OA patients has been reported by Wilson *et al*. since 1998, which are represented by increased signal intensity in fluid sensitive MRI and decreased signal intensity in the T1-weighted sequences[253]. These lesions occur in areas where trabecular bone structure is altered and bone marrow fibrosis takes place, therefore, they cannot be observed on plain radiographs[254]. Compared with non-sclerotic regions, BMELs are appear to be more present in sclerotic areas, with increased bone volume fraction and trabecular thickness[255]. Furthermore, corresponding with BMELs, localized activation of bone repair accompanied with targeted bone remodeling and more cartilage damage has been detected[256-259]. In turn, the damaged cartilage, inflammatory reaction in regard to cartilage degraded products and other micro-traumatic changes associated with altered biomechanics may help to generate BMELs[260]. Numerous researches have demonstrated that BMELs are correlated with the severity of joint pain, cartilage and bone changes in the OA progress[261-266].

1.6.6.1.4 SCB Cysts

SCB cysts, firstly identified in 1940, are not only belonging to OA but can also be found in a few healthy subjects[267-270]. The SCB cysts are composed of fibro-connective tissue that may initially contain fluid but ossify in later stages. Usually, they coexist with BMELs, particularly large BMELs of grade 3 or higher, and recently it was proposed that BMELs can develop into SCB cysts[271-273]. Furthermore, Tanamas *et al*. demonstrated that SCB cysts are associated with more severe structure changes and worse clinical outcomes compared with knees without BMELs or having BMELs

alone[274]. Thus, the SCB cysts may predict an advanced disease state according to their formation by severe BMELs.

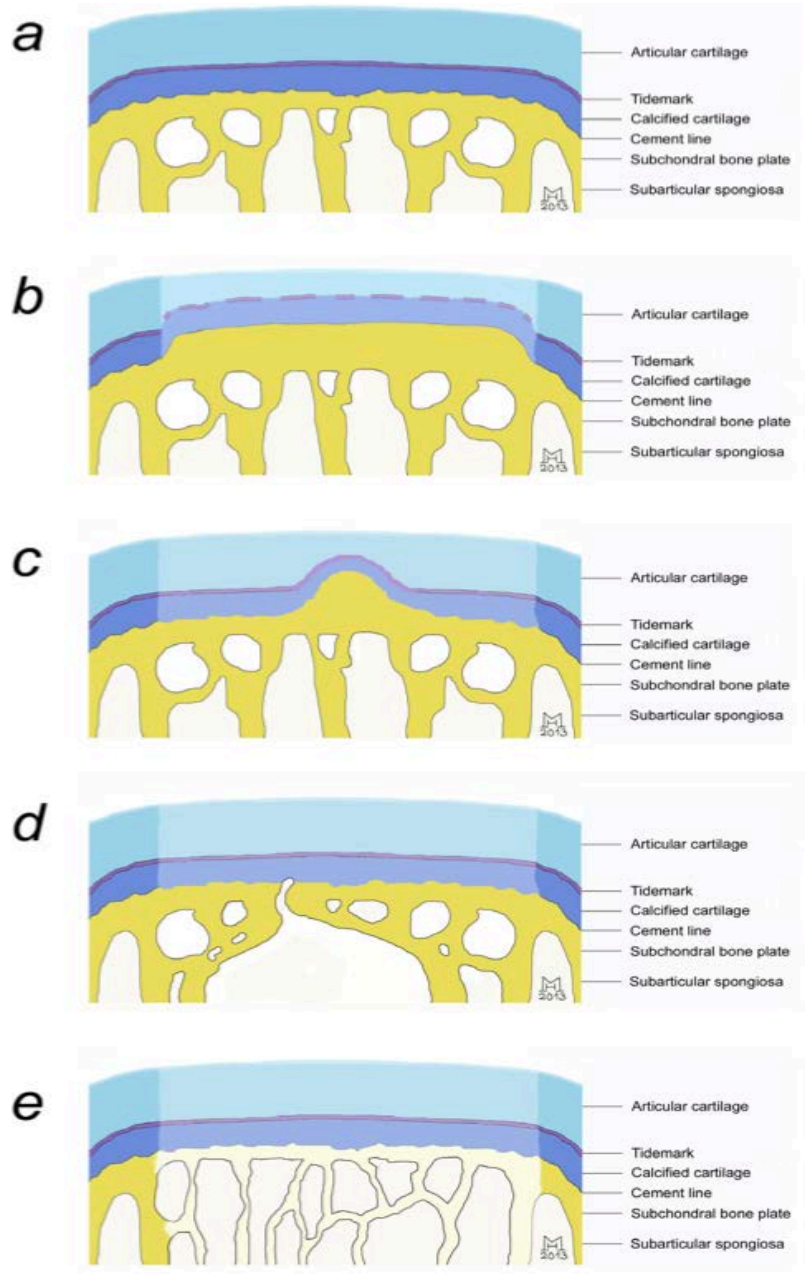

Figure 1.1. 17: The structure alterations of osteochondral unit from normal joint to OA. (a) The normal osteochondral unit. (b-e) the complex alterations of the subchondral bone during osteochondral repair, characterized by (b) the generalised upward migration of the subchondral bone plate, (c) the formation of focal intralesional osteophytes, (d) the appearance of subchondral bone cysts, and (e) the impairment of the microarchitecture of the subchondral bone. (P Orth *et al*. 2013)

1.6.6.2 Potential molecular mechanism of SCB pathology

The characteristics of pathological OA SCB described above impel researchers to make efforts to better understand the underlying mechanisms in the aim of contributing to the development of future therapeutics. During the last two decades, several factors such as WNT, TGF-β/BMP and OPG/RANK/RANKL, IGF-1 and Hepatocyte Growth Factor (HGF) have been proposed to be involved in SCB pathology during OA.

1.6.6.2.1 WNT

The wingless type (WNT) signaling pathway is crucial for early development, organogenesis and for maintaining tissue homeostasis[275]. It is divided either into a canonical route with the involvement of the protein β-catenin or into a non-canonical pattern, which is protein β-catenin independent[186]. *FRZB* gene coding for an antagonist of WNT, namely the secreted Frizzled-related protein 3 (sFRP3) was identified in OA. Polymorphisms of this gene, inducing a loss of function of sFRP3, could be detrimental for the joint suggesting elevated WNT in OA[276-278]. The phenomenon mentioned above in human being is consistent with that found in animal models, $Frzb^{-/-}$ mice are more prone to OA with more severe cartilage damage and increased bone formation compared with wild-type controls[279]. Dickkopf (DKK) 1 and 2 also served as WNT signaling antagonists. Though DKK1 and DKK2 were mostly reported to be present in cartilage with DKK1 also found in human OA synovium, they influence the osteoblast characteristics. DKK1 regulates osteogenesis while DKK2 is involved in osteoblast proliferation, terminal differentiation and mineralization[280-285]. Furthermore, it is reported that DKK1 increased while DKK2 decreased in OA cartilage compared with normal[284], but it is worth to mention that the DKK2 levels are induced by TGF-β which is produced by OA osteoblasts in SCB[286]. Considering DKK1 and DDK2 roles in controlling osteoblast phenotype combined with elevated WNT signaling in OA, it may explain, at least partly, the osteophyte formation and hypo-mineralization in OA SCB. Conversely, the secretion of WNT agonists in joints such as WNT-inducible signaling pathway protein 1 (WISP1) and WNT 16 will favor the OA cartilage degradation and SCB remodeling[186].

1.6.6.2.2 TGF-β/BMP

The TGF-β superfamily is composed of more than forty cell regulatory proteins, such as TGF-βs, BMPs, Nodal and Activin[287]. TGF-β, whose concentration is higher in OA than in normal samples, promotes cartilage repair in the early stage of OA due to the ability of stimulating matrix synthesis[288,289]. For SCB, as mentioned above, TGF-β contributes to the osteophyte formation and abnormal mineralization in OA. In addition, injection of TGF-β into mice joint will induce OA-like changes, while ablation of TGF-β during experimental OA prevents osteophyte formation[186,290,291]. On the other side, BMPs play a significant role in promoting extracellular matrix synthesis such as proteoglycans and facilitate cartilage repair. However, they can also participate in the chondrocyte terminal differentiation by inducing MMP-13 expression, which is responsible for type II collagen degradation[289,292,293]. The role of BMP signaling in OA progress cannot be ruled out, as reduced BMP levels are associated with decreased SCB density and disturbed collagen fiber arrangement, as observed in mice[294]. The connection between TGF-β1 and BMP signaling means a lot for the osteoblast differentiation. Indeed, their combination enhances ectopic bone formation compared to effect of each alone whereas the TGF-β/BMP knock-out models demonstrated the defect in bone formation[295-297].

1.6.6.2.3 OPG/RANK/RANKL

All belonging to TNF superfamily, osteoprotegerin (OPG) and the receptor activator of NF-κB ligand (RANKL) are produced by osteoblasts whereas RANK, the receptor of RANKL is localized on osteoclasts, constitute a molecular triad[243,298]. Though the molecular triad can be seen in chondrocytes and contributes to the cartilage pathophysiology, it is more implicated in OA SCB metabolism[299-302] (Figure 1.1.18). Normally, RANKL is expressed in either membranous or soluble forms and regulates the formation and activation of osteoclasts in bone remodeling by binding with RANK[303]. OPG protects the skeleton from excess bone resorption by binding to RANKL and preventing its binding to RANK[304]. The balance between OPG and RANKL plays an important role in the bone pathophysiology and an altered OPG/RANKL ratio has been identified in OA. It is reported in human OA SCB that osteoblasts expressing a low OPG/RANKL ratio induce more mature osteoclasts than those expressing a high OPG/RANKL ratio, which suggests the existence of both bone

resorption and formation[298,305]. Thus, the OPG/RANKL ratio may indicate the OA stage in which SCB is involved.

In addition to the signaling pathways and factors described above, other factors involved in the control of bone remodeling in OA deserve to pay attention. Genetic and epigenetic changes have been identified in OA patients and animal models[306-309]. A number of differentially expressed genes have been identified by gene microarray analysis, such as elevated mRNA for ALP, osteocalcin, osteopontin, COL1A1 and COL1A2, decreased mRNA for IL-6 and IL-11[310,311]. The vascular pathology is involved in OA SCB remodeling as well, which is represented by the impaired venous blood flow. During OA, venous stasis or occlusion which will result in intra-osseous hypertension may decrease the bone blood perfusion and subsequently construct an ischemia condition leading to bone death[312].

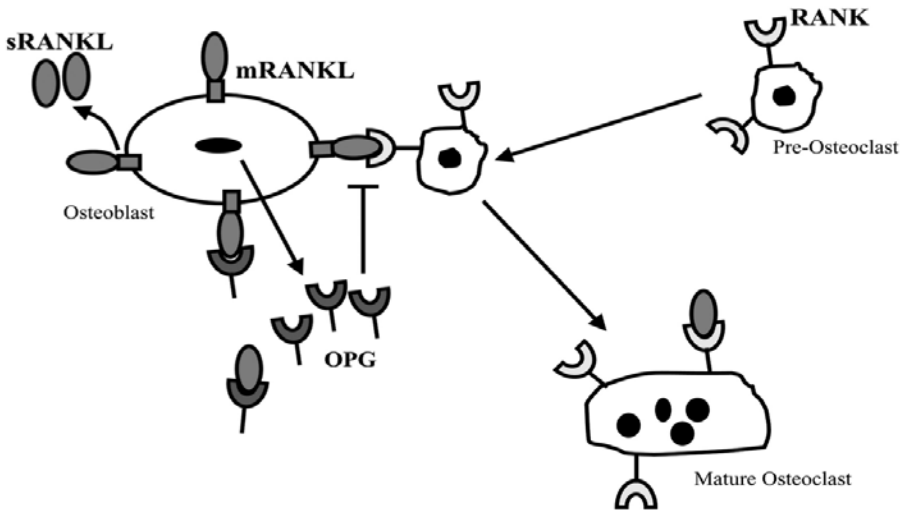

Figure 1.1. 18: OPG/RANK/RANKL molecular and cellular mechanisms of action. RANKL is produced by the osteoblast cells in membranous (mRANKL) or soluble (sRANKL) forms. (Tat KS *et al*. 2009)

1.6.6.3 The interplay between articular cartilage and SCB in OA

Physically, the calcified cartilage offers a transitional area between overlying cartilage and beneath SCB, the porous SCB plate permits the formation of channels which are responsible for the nutrition and metabolism products exchanges due to the penetrated vessels. During OA, the increased vascularization and the development of microcracks in the bone matrix reinforce the interaction between cartilage and SCB, as more penetrated channels allow for the exchange areas[313,314]. More or less, an interaction

between articular cartilage and SCB in OA has been described. Henrotin Y *et al*. has designed a figure describing the hypothetical crosstalk between cartilage and subchondral bone in OA (Figure 1.1.19).

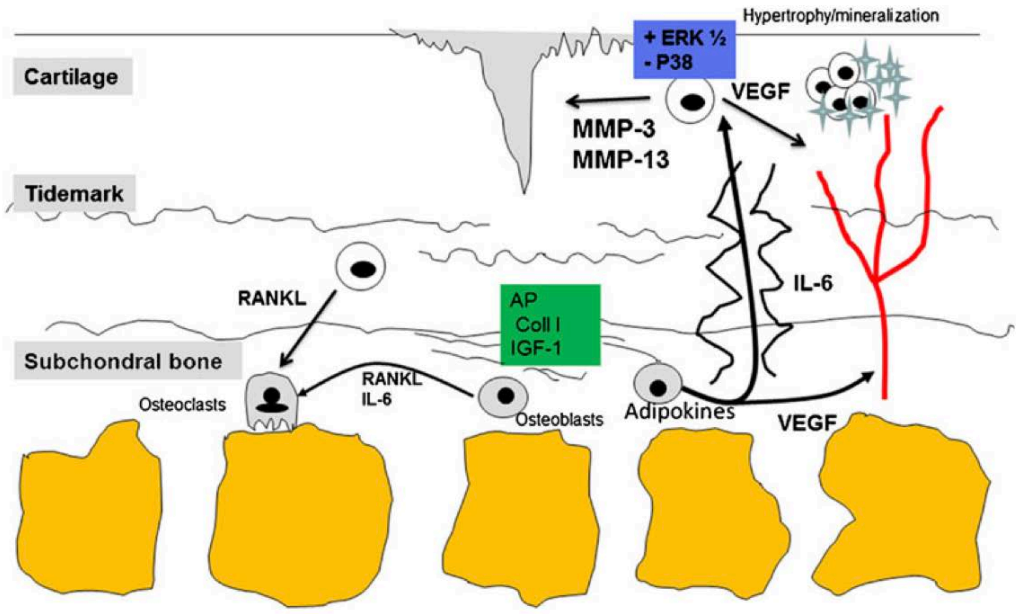

Figure 1.1. 19: Hypothetical crosstalk between cartilage and subchondral bone in OA. (Henrotin Y *et al*. 2009)

In OA patients, SCB thickening and BMELs are found to be followed by cartilage thinning. Whether co-culture SCB osteoblasts with medium conditioned by OA articular chondrocytes or chondrocytes with medium conditioned by OA SCB osteoblasts, the aggrecanase and collagenase expression is stimulated[35,186,315,316]. In animal models, the inhibition of bone resorption results in a reduction of cartilage degradation[317,318]. Even in a bovine explant culture, survival of chondrocytes is influenced by SCB[319]. Numerous factors are considered to mediate the interactions between the articular cartilage and SCB in OA. For instance, IGF-1 and TGF-β, which are growth factors for SCB, are secreted in higher amounts by deteriorated cartilage, will partly account for the bone sclerosis[320]. In addition, the presence of low ratio OPG/RANKL has been reported in OA cartilage[299,321]. OPG, which is mainly produced by osteoblasts, can inhibit chondrocyte apoptosis by blocking the interaction of RANKL with TRAIL[322]. It is worth to mention that the increased expression and production of HGF has been detected in OA SCB, while only HGF protein is present accompanied with induced MMP-13 expression in the intermediate and deep zone of OA cartilage[236,323,324]. In addition, SCB is responsible for absorbing much more

mechanical loading than cartilage. Therefore the alterations of SCB will influence the distribution of the bearing load[256,325]. In the late stage of OA, increased bone density with reduced mineral content renders the SCB more susceptible to deformation resulting in the alleviation of its role in mechanical loading, which in turn transfers more loading pressure to the overlying damaged cartilage leading to a more severe degradation[256,326,327].

2 Galectin-3

2.1 Galectin superfamily

Previously termed S-type lectins, galectins are a superfamily of β-galactoside binding animal lectins. They are widely distributed in various human tissues as well as in other species, including sponges, fungi, insects and even viruses[328]. Galectins have been shown to play an important role in cell-cell and cell-matrix interactions and recently their immunoregulatory roles are more and more emphasized[329,330]. Until now, 15 galectins have been indentified in mammals, with 12 galectins present in humans[329,331]. According to the numbers and the organization of carbohydrate recognition domains (CRD) which are highly conserved in galectins with approximately 130 amino acids, the galectin family is divided into three subgroups (Figure 1.1.20): Prototypic galectins (galectin-1, -2, -5, -7, -10, -11, -13, -14, and -15) contain one CRD but can form homodimers; Tandem repeat type galectins (galectin-4, -6, -8, -9 and -12) consist of a single polypeptide chain that forms two distinct but homologus CRDs, which are bridged by a small peptide domain; Chimera type only represented by Galectin-3 which contains one CRD connected to a N-terminal domain (ND)[331,332]. The most studied of the galectins is galectin-3, which has been shown to be associated with various diseases, such as thyroid cancer, tumor metastasis, asthma, heart failure and other inflammatory processes[329,331,333-338]. Thus, it deserves to investigate its structure and function in pathophysiological conditions, thereafter, the attempt to treat diseases based on the findings could be realized.

2.2 The structure of Galectin-3

Firstly named as Mac-2, galectin-3 was initially identified in mouse thioglycolate-elicited peritoneal macrophages with molecular mass at 32-kDa. Until 1994, when the unified standard nomenclature was constructed, numerous names have been given to this unique chimera galectin in reference to the locations and species. For instance, galectin-3 was previously described as ε-BP and L-34 in basophilic leukemia cells and embryonic fibroblasts, respectively, while in human lung tissue it was called HL-29 and in mouse fibroblasts, CBP-35[339-341].

Correspondingly, the molecular mass ranges from 29- to 35-kDa due to the location and species. It is formed by a single polypeptide chain with a N-terminal domain and a C-terminal CRD, both of them devoted to the characteristic and function of galectin-3. Ubiquitously expressed in adults, galectin-3 has been found in brain, kidney, liver, skeleton and other organs[342,343].

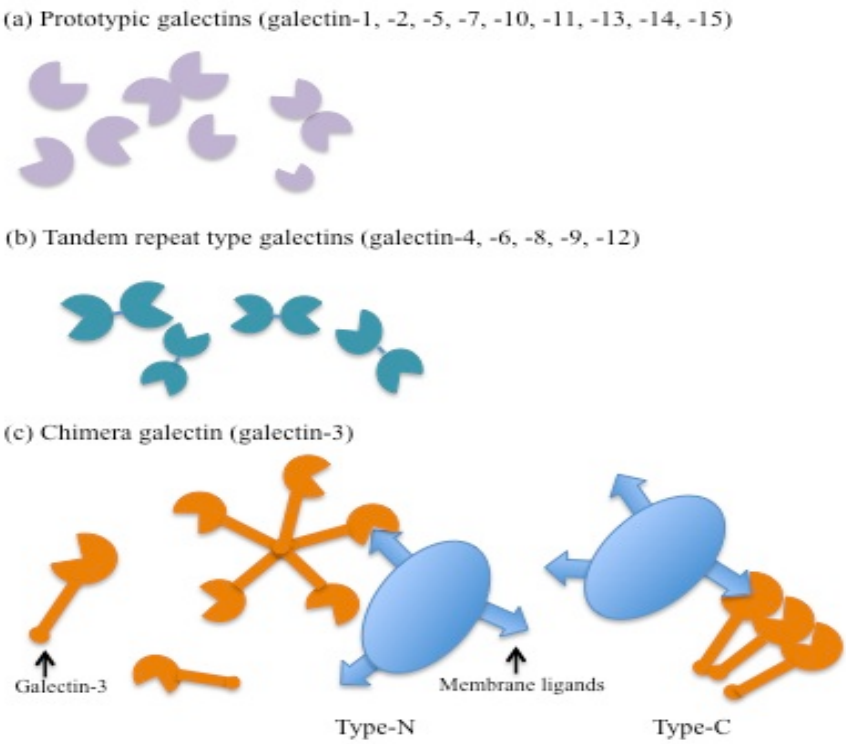

Figure 1.1. 20: Structure and classification of the galectin family. (adapted from Blidner AG *et al*. 2013 and Lepur A *et al*. 2012)

In human, the gene coding galectin-3 is located in chromosome 14q21-22 and named *LGALS3*[344]. It is composed of six exons and five introns[345,346](Figure 1.1.21). In humans, exon III is responsible for the ND translation while exon V is in charge of the CRD translation[347,348]. However, though the exon responsible for the production of ND is similar in mouse and human, exons IV, V and VI are coding for the CRD production[349]. The ND of galectin-3 consists of 100-150 amino acid residues, including a repetitive sequence of proline, glycine, tyrosine and glutamine[339,347,348,350,351]. In addition, a casein kinase I serine phosphorylation site has been found in the initial 12 amino acid sequence[352-354]. ND is important for the oligomerization and secretion of galectin-3. Due to the collagen-like sequence in ND, it can be cleaved by MMP-2 and MMP-9 at the position Ala62-Tyr63[355]. The cleaved galectin-3 shows higher affinity to the carbohydrate ligands compared with intact

protein, though devoided of its biological properties due to the lack of ND[332]. However, Hirabayashi *et al*. showed that ND enhanced the galectin-3's affinity towards basic recognition unit such as Lac or LacNAc[356]. The other part of galectin-3 is CRD, which contains about 130 amino acid residues to form a globular structure, accounting for the lectin activity of galectin-3[340,357]. This CRD can specially bind to laminin poly-N-acetyllactosamine residues. A NWGR motif has been found in CRD, which is also present in the BH1 domain of Bcl-2 family proteins, is responsible for the anti-apoptotic activity of galectin-3[358]. In addition, not only ND but also CRD can benefit the oligomerization of galectin-3, called type-N and type-C[359].

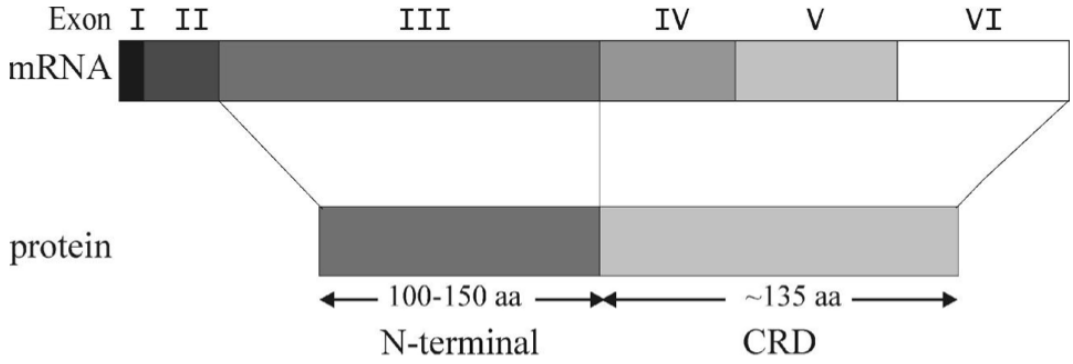

Figure 1.1. 21: The organization of the mRNA and the structure of human Galectin-3. (Krzeslak A *et al*. 2004)

2.3 The secretion of galectin-3 and its ligands

Not only present in cytoplasm and nucleus, galectin-3 is also found in extracellular matrix. Since there is no signal sequence for galectin-3 secretion, a non-classical secretion mechanism rather than endoplasmic reticulum/Golgi is proposed[360,361]. Numerous researches reported that the first 11 amino acids in ND are important for the galectin-3 secretion, since removal of them will prevent the export of the protein[330,362-364]. Recently, Shalini *et al*. find that calpain-4 modulates the phosphorylation status of galectin-3 on tyrosine residues 79, 107 and 118, which will indirectly influence its secretion[365]. Despite the mechanism of secretion is not revealed yet, ectocytosis seems to be involved[350,361,366]. For this type of secretion, galectin-3 is synthesized in cytoplasm and accumulated under plasma membrane to form vesicles outward[350,362,364,367]. This process is a rate limiting step for galectin-3 secretion, which is favored by heat shock proteins (HSPs) and calcium

ionophores[361,362]. The next step for galectin-3 secretion is the vesicles pinch off evaginating membrane to be released into extracellular matrix, the vesicles preventing galectin-3 from proteolysis until breakdown by factors released by cells[362,364] (Figure 1.1.22). The secretion of Gal-3 varies on cell types which could be facilitated by the interaction with membrane lipids in an energy-independent manner[367]. In non-polarized cells, Gal-3 is transported through acidified endosomal compartments bound to vesicular carriers[368].

Owing to the affinity to glycan structure, galectin-3 binds with glycoconjugate ligands. The binding affinity is relative to the multivalency and oligomeric state of galectin-3. It is also influenced by the multivalency of the ligands. Galectin-3 shows a weak affinity to simple glycans such as disaccharides or trisaccharides, but its affinity towards glycans with repeating [-3Galβ1-4GlcNAcβ1-]$_n$ or poly-N-acetyllactosamine sequences is higher[330]. The enzyme β1,6-N-acetylglucosaminyltransferase V (Mgat5), which transfers β1, 6-N-acetylglucosamine to a longer and more branched chain with N-linked and O-linked oligosaccharides, maybe responsible for the modification of glycoconjugate ligands leading to the indirect influences on galectin-3's function[369-371]. According to the location, ligands of galectin-3 can be divided into extracellular, cell membrane and intracellular sections. The extracellular ligands include laminin, fibronectin, tenascin, Mac-2 binding protein, prostatic acid phosphatase (PAP), zinc alpha-2-glycoprotein (ZAG), dipeptidyl peptidase-4 (CD26), aminopeptidase N (CD13)[372-375 376]. Cell membrane ligands consist of integrin, CD98, Lamp 1, and MP20[377-379]; while intracellular ligands are represented by cytokeratins, CBP70, Chrp, Gemin4, Alix/AIP-1 and Bcl-2[380-383]. It is well known that galectin-3 interacts with its ligands in a lectin-glycoconjugate manner, but its interaction with intracellular ligands bypasses a protein-protein pattern[1,380]. By binding with its ligands, galectin-3 exerts physiological and pathological roles.

2.4 The function of galectin-3

The function of galectin-3 depends on its cellular localization (Figure 1.1.23). According to various ligands described above, binding with them defines the intracellular and extracellular role of galectin-3.

Intracellularly, due to the presence of the special sequence similar to Bcl-2, a well characterized suppressor of apoptosis, galectin-3 is suggested to be implicated in the regulation of apoptosis[384]. In fact, cytoplasmic galectin-3 demonstrates an anti-apoptotic activity. Furthermore, galectin-3 binds to Bcl-2 in a lectin-glycoconjugate manner, since the interaction can be inhibited by lactose[358]. Other molecules such as CD95, Nucling and Alix/AIP1 are also reported to interact with galectin-3 in contribution to the apoptosis regulation[385-387]. The cytosolic galectin-3 is also implicated in cell proliferation, differentiation, survival and death, in which the K-Ras protein and Akt protein are conerstones[388-391]. Galectin-3 can be found in nucleus as well. Compared with unclear extracellular export mechanism, the import of galectin-3 into nucleus is less elucidated, but the first 11 amino acids in ND are considered to implicate in both[363]. In addition, recent studies also proposed the regulatory role of nuclear galectin-3 in Wnt/β-catenin signaling pathway, because β-catenin was identified as a novel binding partner of galectin-3 in nucleus[392,393].

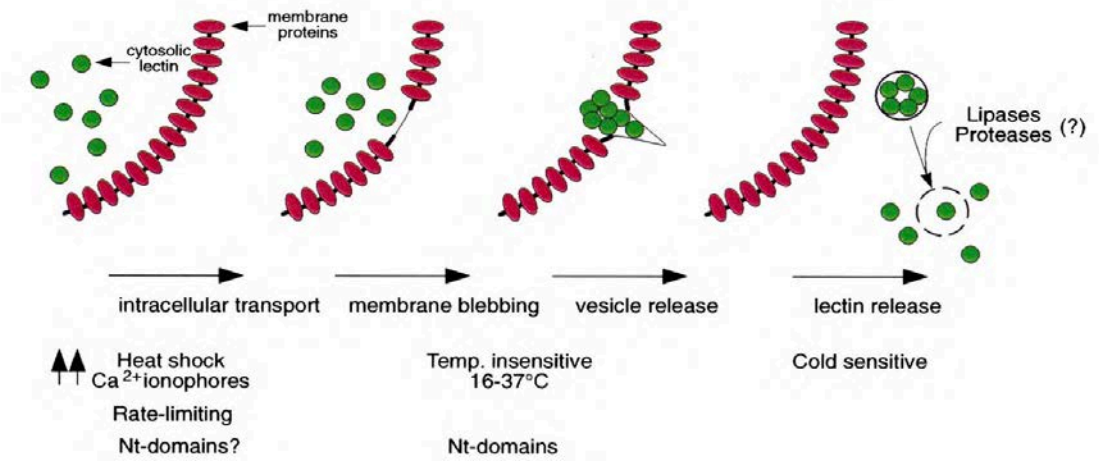

Figure 1.1. 22: The potential secretion mechanism of galectin-3: vesicle ectocytosis. (Hughes RC *et al*. 1999)

Though containing a Bcl-2 like sequence, extracellular galectin-3 induces apoptosis in human T cells, which involves cell surface glycoproteins including CD7, CD29, CD45 and CD71[378,394,395]. However, the function of galectin-3 in ECM seems to be a mediator in cell adhesion, cell activation and a chemoattractant. Galectin-3 modulates cell adhesion through the interaction with its ligands located not only in ECM, but also on the membrane surface, where integrins served as main ligands[332]. This modulation can be positive or negative, mostly depending on the cell types. For example, galectin-3 promotes adhesion of human neutrophils to laminin and to

endothelia cells, but on the other side it disrupts the interaction of thymocyte with the surrounding microenvironment[396-398]. Usually, galectin-3 can cross-link membrane ligands triggering a serial of biochemical reactions in cells, thus resulting in the activation of these cells, particularly those implicated in immune reactions (Figure 1.1.24)[332]. In contrast to its common cell activation role, galectin-3 is also reported to suppress the myeloid cells activation[399,400]. Finally, galectin-3 acts as a chemoattractant for monocytes and macrophages. Both ND and CRD are indispensible for this activity[401]. This chemotaxis is concentration dependent as at high concentration of galectin-3 (1μM) chemotactic effects are obtained, while at lower concentrations (10-100 nM) chaotic cell motion were demonstrated[332].

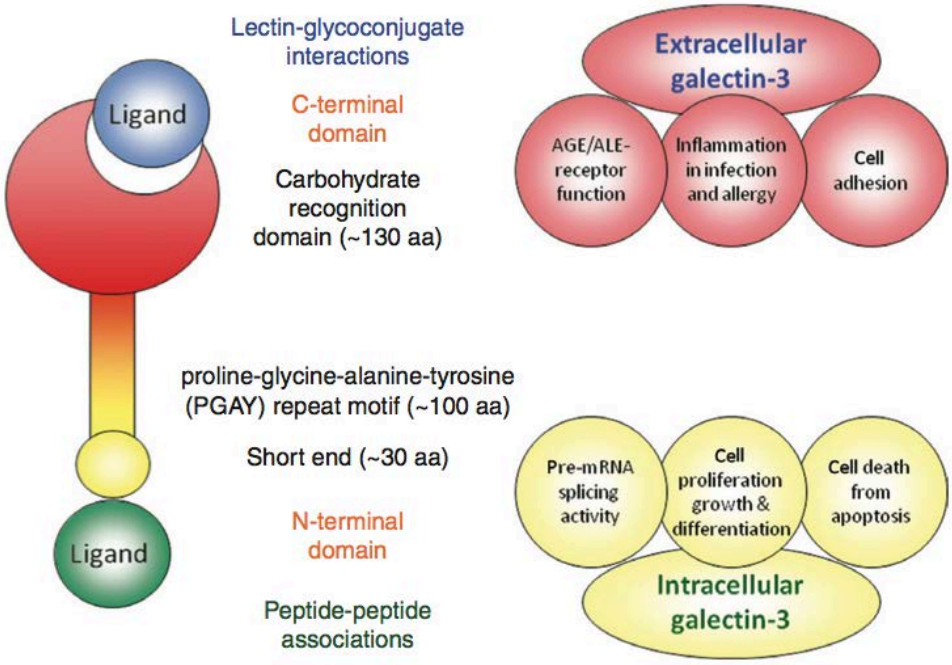

Figure 1.1. 23: Galectin-3 structure and function. (Pugliese G *et al*. 2014)

2.5 The involvement of galectin-3 in various diseases

The ubiquitous localization of galectin-3 suggests its importance in contribution to biological processes. Through interacting with numerous ligands, galectin-3 is not only involved in physiological conditions, but also implicated in pathological conditions. The presence of galectin-3 in cancer, heart failure, fibrotic diseases, diabetes, arthritis and other diseases has been reported. In contrast to the normal presence, the modified concentration and localization of galectin-3 suggest its prominent role in the initiation and/or progression of the diseases.

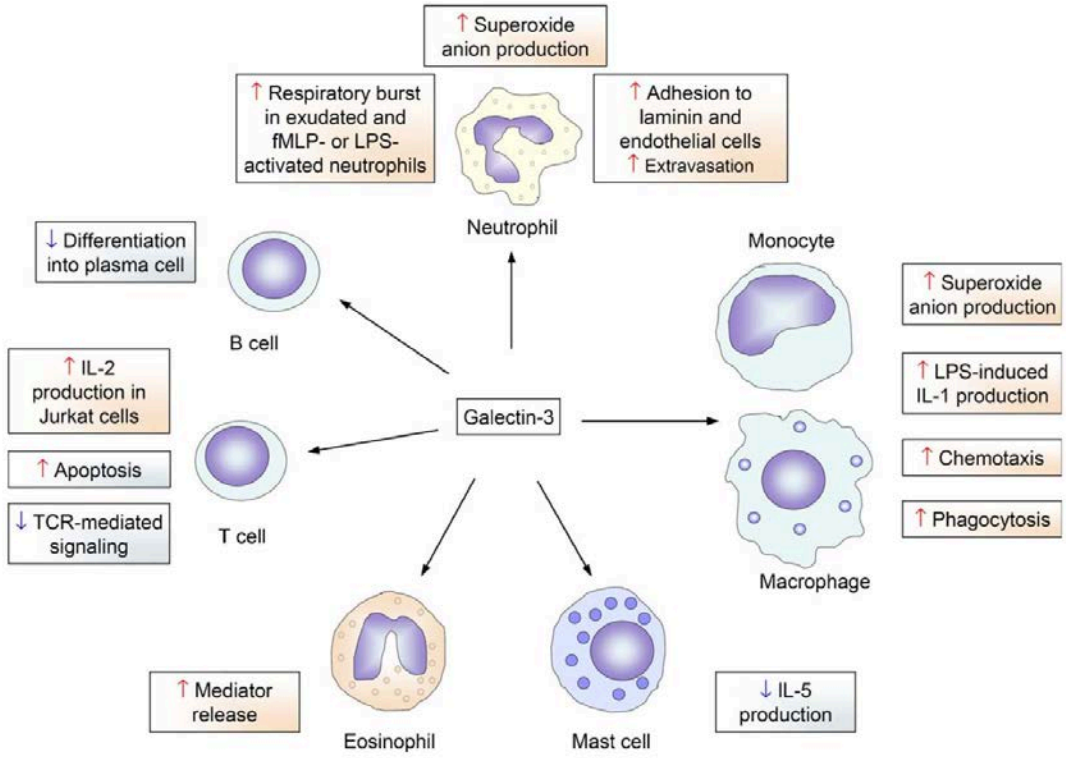

Figure 1.1. 24: The effects of galectin-3 on immune cells. Red upwards arrows indicate positive effects, blue downwards arrows indicate negative effects. IL-1, -2, -5: interleukin-1, -2, -5, LPS: lipopolysaccharide, fMLP: formyl-methionyl-leucyl-phenylalanine, TCR: T cell receptor. (Dumic J *et al*. 2006)

Until now, galectin-3 is considered to be associated with the progression of thyroid cancer, lung cancer, breast cancer, gastric cancer, pancreatic carcinoma…[337] Accumulated evidence indicate the role of galectin-3 in tumor cell transformation, migration, invasion and metastasis (Figure 1.1.25). The intact structure ensures galectin-3's activity in cancer process, as the lack of either CRD or ND triggered reduced tumor cell adhesion and aggregation as well as tumor growth and metastasis respectively[402,403]. In addition, Mgat-5 has been reported to be upregulated in multiple cancer types[404-407]. As described before, rely on the capacity of generating high affinity ligands of galectin-3, the Mgat-5 induction in cancer leads to more interactions between galectin-3 and its ligands, which indirectly suggests that the galectin-3 activity is associated with cancer progression. In thyroid cancer, elevated galectin-3 expression has been observed. This condition is deficient in normal thyroid tissue and rarely occurs in benign thyroid lesions. Thus, galectin-3 is proposed clinically to be applied as a diagnosed marker for thyroid cancer and possibly targeting galectin-3 may improve the outcomes of patients[408]. Though galectin-3

promotes most cancer progression, it can be also a suppressor. For instance, reduced expression of galectin-3 is supposed to be involved in transferring benign prostate gland to hormone-refractory malignant disease[337].

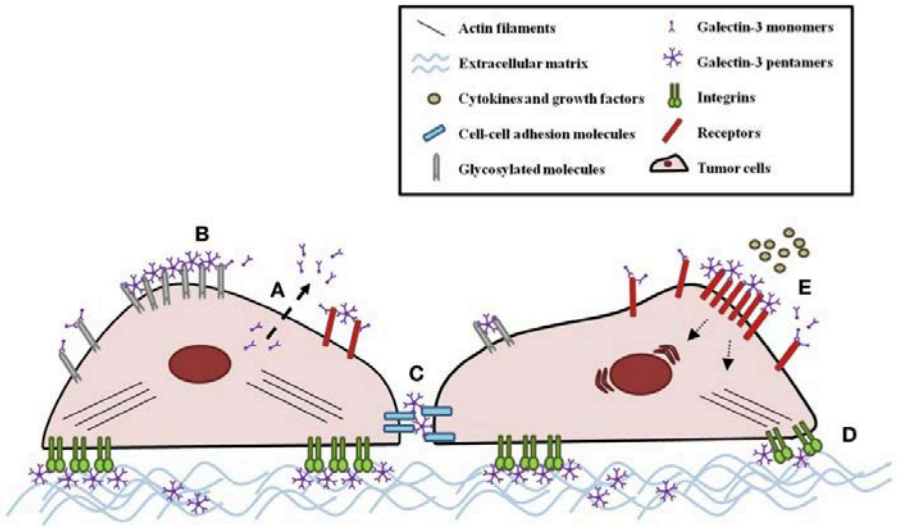

Figure 1.1. 25: Galectin-3 modulates tumor cell behavior. (A) Galectin-3 monomers are secreted by a non-classical mechanism. (B) Galectin-3 constructs a complex called lattice by binding with its receptors on cell membrane to modulate (C) cell adhesion and (D) migration. (E) The galectin-3-glycan lattice also can modulate the reponse of cell to cytokines and growth factors. (Fortuna Costa A *et al*. 2014)

In the past decades, galectin-3 has been proposed as an important factor in contribution to heart failure (HF)[409-411]. The potential mechanism by which galectin-3 promotes HF development has not been clearly elucidated yet. However, with animal models, it is proposed that galectin-3 is released by macrophages and fibroblasts, into myocardium in case of damage or ageing. This secretion leads to myofibroblasts proliferation and procollagen-1 deposition resulting in cardiac fibrosis which is considered as a hallmark of cardiac remodeling and HF[412-414]. Others also proposed the contribution of galectin-3 in the aldosterone-induced vascular fibrosis[415,416]. Galectin-3 is applied as a diagnosis biomarker for acute HF and outcome predictor for chronic HF[338]. Furthermore, several epidemiologic studies point out that galectin-3 not only serves as a prognostic role, but also predicts the risk of mortality[417-419]. Although the importance of galectin-3 in HF has been emphasized, it is difficult to establish its independent prognostic role, while the combination of galectin-3 and N-terminal pro brain natriuretic peptide (NT-ProBNP) seems be more efficient than either of the two markers separately in predicting HF and relative mortality[420-422].

In addition to cancer and HF, galectin-3 is also involved in fibrosis disease, asthma, arthritis, diabetes and others. Though the mechanism of these diseases is not clearly interpreted, more or less, galectin-3 seems to act as a pro-inflammatory factor in these pathological conditions. The association of galectin-3 and arthritis will be discussed below in the review.

3 Review of Gal-3 and arthritis

Galectin-3: a key player in arthritis

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Abstract

Arthritis is more and more considered as the leading reason for the disability in the world, particularly its main entities: rheumatoid arthritis and osteoarthritis. The common feature of arthritis is inflammation, which is mainly supported by the synovitis (synovium inflammation). During the inflammatory phase of arthritis, many pro-inflammatory cytokines and mediators are secreted into the joint, which are responsible for cartilage degradation and excessive bone remodeling. Amongst them, a β-galactoside-binding lectin, galectin-3, has been reported to be highly expressed and secreted by inflamed synovium of rheumatoid arthritis and osteoarthritis patients. Furthermore, galectin-3 has been demonstrated to induce joint swelling and osteoarthritis-like lesions *in vivo*. However, the mechanisms underlying its pathophysiological role in arthritis have not been fully elucidated. This review will deal with the characterization of arthritis and galectin-3 and summarize our current knowledge of the contribution of galectin-3 to joint tissue lesions in arthritis.

Key words: galectin-3, rheumatoid arthritis, osteoarthritis, synovitis

1. Introduction

Arthritis composed mainly of rheumatoid arthritis (RA) and osteoarthritis (OA) is accompanied by pain, inflammation and stiffness of the joints, and causes disability of many people all around the world. In France the prevalence of radiographic OA was estimated of about 17% and a recent study indicates that the prevalence of symptomatic knee OA is around 2% for both males and females aged of 40 to 49 years, while increasing to 10 to 15% for people of 70-75 years old [1]. In contrast, the RA prevalence is 0.3% in France [2].

The aetiology of each disease is different, as RA is an autoimmune disease while OA is a degenerative/mechanical one. Although synovitis is common in OA, it is less inflammatory than in RA and scarcely forms pannus, a characteristic of RA [3]. Synovitis induces alterations of cartilage and subchondral bone (SCB) remodeling. At the pannus level, where leukocyte infiltration occurs beside synovitis proliferation, pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are secreted into the joint. Another soluble factor, galectin-3 (gal-3), is markedly present in synovial tissue during the inflammatory flares. Of note, compared to OA, gal-3 concentration found in RA synovial tissue is higher [4]. In addition, genetic polymorphisms of galectin-3 may influence the susceptibility to RA, as the galectin-3 gene allele (LGALS3 +292C) is more prevalent in RA patients than in healthy controls [5].

In this mini-review, we summarise some properties of gal-3 and present available data supporting its pathophysiological role on the different joint tissues (synovium, cartilage and subchondral bone) during arthritis.

2. Galectin-3: a peculiar member of the galectin family

Galectins are a family of evolutionary conserved animal lectins which shared close sequence homology in their carbohydrate-recognition domain (CRD). Another criterion of these lectins is their affinity toward β-galactosides. To date, 15 galectins have been discovered and classified into three distinct subgroups based on the number and on the organization of CRDs (Fig 1).

Prototypic galectins (galectin-1, -2, -5, -7, -10, -11, -13, -14, and -15) contain one CRD and are able to homodimerize. The prototypic galectin-1 was one of the most extensively pro-tumorigenic galectin studied. Its concentration in blood has been demonstrated to correlate with cancer progression in a number of studies. More interestingly, this correlation can be disrupted by injection of therapeutic molecules able of blocking the CRD activity [6].

Tandem repeat type galectins (galectin-4, -6, -8, -9 and -12) consist of a single polypeptide chain that forms two distinct but homologus CRDs, separated by a linker up to 70 amino acids.

Gal-3, which has been called Mac-2 when first discovered in macrophages, is the unique chimera type in the family of galectins. It was found to be widely distributed in tissues, including the gut, brain, kidneys and skeleton [7]. Probably as the best studied member of the galectin family, gal-3 can amplify inflammatory responses [8] and plays key roles in several physiologic and pathologic processes including angiogenesis [9] and tumor development and progression [10].

2.1. Galectin-3 synthesis and release

The gene coding gal-3 was mapped on human chromosome 14 (14q21-22) and named *LGALS3* [11]. The protein is composed of three structural motifs including a short amino terminal region of 12 amino acids, a collagenase-sensitive sequence rich in G-X-Y tandem repeats that are typical of the collagen supergene family, and a carboxy-terminal half containing the globular CRD.

Though gal-3 lacks a signal peptide, it can be secreted via a non-classic pathway, namely ectocytosis [12]. In this secretion pathway, cytosolic gal-3 concentrates under the plasma membrane and constitutes aggregates that are then included in evaginating protrusions (blebs) of the membrane. Then, those blebs release the soluble gal-3 into the extracellular space (Fig 2). The secretion of gal-3 depends on cell types and could be facilitated by its interaction with membrane lipids in an energy-independent manner [13]. In non-polarized cells, gal-3 is transported through acidified endosomal compartments bound to vesicular carriers [14]. Whatever the secretion type is, residues 89 to 96 in the N-terminal domain including proline residues are indispensable [15].

2.2. Galectin-3 binding properties

Most functions of gal-3 are supported by the binding of its CRD to glycosylated structures. The affinity of galectins increases if galactose is coupled to other saccharides, particularly when forming N-acetyllactosamine, which is recognized as the preferential ligand of gal-3 [16]. In addition to this lectinic recognition of the ligand, gal-3 must multimerize via its N-terminal domain (type-N self-association) to generate biological activities. However, a proteolytic cleavage of gal-3 in

the collagen-like domain increases its binding affinity for the ligands but does not induce a cell response. Few years ago, a Swedish group demonstrated that whole gal-3 could alternatively multimerize via the CRD, which also resulted in an increased affinity for the ligands. They named this phenomenon, type-C self-association [17].

To date, identified gal-3 ligands include laminin [18], fibronectin [16], hensin [19], elastin [20], collagen IV [21], integrin β1 [22]. A recent report also proposed other ligands of gal-3 such prostate-specific antigen, aminopeptidase N, prostatic acid phosphatase, zinc alpha-2-glycoprotein and dipeptidylpeptidase-4 [23].

2.3. Galectin-3 localization and functions

By binding to its ligands and according to its localization, gal-3 exerts its effects in a carbohydrate-dependent or -independent manner.

Intracellularly, gal-3 shuttles between the nucleus and cytoplasm, being engaged in a variety of cellular processes, such as cell proliferation, differentiation, apoptosis, cytokine secretion, RNA splicing and protein traffic. For instance, gal-3 can protect T cells, macrophages and breast carcinoma cells from apoptosis [24,25]. Its anti-apoptotic activity comes from the presence of a NWGR motif within the CRD and which is highly conserved within the BH1 domain of Bcl-2 family proteins. In addition, phosphorylation of gal-3 at position Ser^6 was also considered essential for the anti-apoptotic activity.

Extracellularly, gal-3 has been demonstrated to be involved in apoptosis and in cell-cell or cell-matrix interactions. These characteristics might have relevance for the invasion-metastasis cascade. The formation

of structures, composed galectin-3 and glycan clusters on cell surface, termed lattices, modulates the tumor cell behavior, such as adhesion and migration. These lattices also extend the exposure of receptors on the cell surface, affecting cell response to cytokines and growth factors [26]. Meanwhile, the ability of gal-3 to promote angiogenesis, is a vital process for the continuous tumor growth, which provides a pathway for dissemination of malignant cells [27]. Moreover, extracellular gal-3 can induce apoptosis of T cells, activate several lymphoid and myeloid cells including mast cells and T cells, resulting in production of reactive oxygen species (ROS), cell degranulation, and cytokine production [8]. The action of extracellular gal-3 on neutrophils is controversial since both apoptotic and anti-apoptotic effects were noticed [28,29]. Furthermore, a deleterious role of gal-3 in joint tissues has been demonstrated in mice, in contrast to its intracellular effects [30].

3. Pathophysiological role of galectin-3 in synovitis

The synovial membrane, which is responsible for maintaining normal joint function and homeostasis, is a thin lining layer within the joint cavity. Synovial fibroblasts produce two important lubricating molecules, lubricin and hyaluronic acid which help to protect and maintain the integrity of articular cartilage surfaces in diarthrodial joints. The pathophysiology of both RA and OA is characterized by the presence of increased gal-3 levels in synovium, which play a role in the inflammatory phase in these diseases. During inflammatory phases, the synovial membrane is a source of pro-inflammatory and catabolic products mainly secreted by synovial macrophages although other immune cells, which have infiltrated the membrane, can participate too [31].

3.1. Arthritic patients and cellular data

Compared to the synovium of healthy people, RA or OA patients express and secrete higher levels of gal-3 [4,32]. At the onset of RA, the synovium is gradually infiltrated by neutrophils, $CD4^+$ T-cells, B lymphocytes, plasma cells and macrophages. Neutrophils produce ROS and other metabolites involved in inflammation. Plasma cells secrete autoantibodies, macrophages and $CD4^+$ T-cells activate B cells which themselves activate CD4+ T in a feed-back positive mechanism. In addition, B lymphocytes, as well as macrophages and neutrophils, secrete numerous proinflammatory chemokines and cytokines responsible for the continuous leukocyte infiltration and cell activation. Then the chronic aspect of arthritis takes place and results in the persistence of the pannus, permanent activation of immune cells, and secretion of proinflammatory soluble mediators by synovium, which lead to degradation of cartilage and bone. In RA, about 39% of fibroblast-like synoviocytes are cartilage adhering cells and are producing gal-3 in the synovium [33]. Reciprocally, gal-3 also contributes to the activation of RA synovial fibroblasts [4]. Thus, gal-3 over-expression leads the activated synovial fibroblasts and synovial macrophages to be less sensitive to apoptosis, thus favouring their proliferation and the invasion of cartilage by binding to the cartilage oligomeric matrix protein (COMP), an important component of hyaline cartilage. RA synovial fibroblasts adhere to COMP with a higher efficacy than OA synovial fibroblasts [34]. In turn, this adhesion stimulated the intracellular expression of gal-3, thus generating a positive feed-back loop, which may be involved in the aggressiveness of synovium. Filer and colleagues [35] showed that *in vitro*, RA synovial fibroblasts stimulated by exogenous recombinant gal-3, secreted proinflammatory cytokines

(IL-6, TNF-α) and chemokines (CCL2, CCL3, CCL5, CXCL8) through several Mitogen Activated Protein Kinase pathways such as p38, JNK and ERK1/2. All these factors can induce their own synthesis by activating the synovial fibroblasts and synovial macrophages, which generates an amplification loop of inflammation. A more recent report demonstrated that LPS-induced IL-6 secretion via TLR receptors is down-regulated in the presence of gal-3 siRNA in human synovial fibroblasts. However, gal-3 siRNAs does not affect MMP3 and CCL5 secretion [36]. A series of evidence have shown that gal-3 secretion was elevated at the sites of joint destruction [4,32,34]. More recently, this was also confirmed for juvenile RA [37]. This reinforces the amplifying role of gal-3 in synovial inflammation.

3.2. Lessons from animal models

Wang et al. demonstrated that gal-3 was over-expressed in the synovium of collagen-induced arthritic (CIA) rats, compared to healthy ones [38], suggesting that this protein was involved in the pathogenesis of CIA. This group identified the role of gal-3 by inhibiting its expression with RNA interference technology (shGal-3) before inducing arthritis [38]. The results showed that rats treated with the shGal-3 construct had less severe arthritis assessed at clinical, radiological and histological levels than untreated rats. Indeed, shGal-3 rats had a more moderate leukocyte infiltration, a decrease in the number of T cells, a smaller synovial hyperplasia and a lower density of the synovial micro-vessels. Of note, the decrease in the number of T cells may be explained by the fact that the intracellular gal-3 depletion causes apoptosis of these cells. Nonetheless, the pivotal role of gal-3 involvement in synovitis was demonstrated with gal-3 deficient mice. In gal-3 knock-down (KO) mice,

antigen-induced arthritis (AIA) is less severe than in wild-type (WT) mice [39]. Indeed, synovial hyperplasia was less marked, and serum levels of proinflammatory cytokines (IL-6, IL-17, TNF-α) together with the number of T cells were lower. When exogenous recombinant gal-3 was injected into the peritoneum of gal-3 KO mice, the severity of joint lesions and the level of proinflammatory cytokines production became similar to those observed in WT mice. Taken together, these results show that invalidation of gal-3 improves the symptoms of experimental arthritis, suggesting that this lectin is involved in the pathophysiology of RA. Furthermore, intra-articular injection of gal-3 induced swelling and OA-like lesions in naïve mice [30]. Taken together, these studies proved that the presence of gal-3 was correlated with inflammation of the synovial membrane and severity of joint degradation.

4. Pathophysiological role of galectin-3 in cartilage degradation

Normal articular cartilage is composed of a dense extracellular matrix (ECM) with sparse distribution of chondrocytes, which are responsible for the production of extracellular matrix components. Aggrecan and collagens are two main contents of ECM, the former endows the cartilage with compressive resistant and shock absorbing capability, whereas the latter mainly represented by type II collagen accounts for the tensile property of the cartilage. Along with a variety of glycoproteins and non-collagenous proteins, aggrecan and collagens form a matrix framework to protect the chondrocytes from the harmful effects of excessive loading. Chondrocytes are quiescent in normal adult cartilage, but this condition is disrupted when arthritis occurs. To compensate for the missing ECM, these cells increase the synthesis of matrix macromolecules by producing growth factors. However, when

they failed to restore the tissue, chondrocytes decrease their anabolic metabolism and produce degrading enzymes. The main source of degrading enzymes contributing to cartilage lesions differs from RA to OA. The former takes the inflammatory synovium as the principle source while the latter puts quickly the stimulated chondrocytes on a prominent position. These enzymes including collagenases and aggrecanases belong to the MMP and the ADAMTS (A Disintegrin And Metalloproteinase with Thrombospondin Motifs) families. The matrix degradation in early OA may be due to proteoglycan loss induced by MMP3 and ADAMTS5 or 4, depending on species. Afterwards, the collagenase activity will increase, especially MMP13, which is highly efficient in degrading type II collagen. Once this collagen network is degraded, the cartilage reaches a pathological step that cannot be reversed.

4.1. Intracellular galectin-3 and chondrocyte data

Colnot et *al* identified the expression of gal-3 in differentiated chondrocytes with a higher concentration in the cytoplasm of mature and early hypertrophic chondrocytes than in the late hypertrophic ones [40]. In the late stage of long bone development, gal-3 null mutant mouse embryos have a decreased number of hypertrophic chondrocytes accompanied with an increased number of empty lacunae and condensed chondrocytes at the chondro-vascular junction. The up-regulation of gal-3 in hypertrophic cartilage has also been reported both in chicken [41] and during the first trimester of human embryogenesis [7]. In OA, an intracellular induction of gal-3 in chondrocytes was reported, as well as an increase of its secretion [42]. These variations of the intracellular content of gal-3 indicate its roles in chondrocyte physiology. For example, it was suggested that gal-3 could be involved in the chondrocyte death and the

vascular invasion coupling process during endochondral ossification [43]. The increase of gal-3 in chondrocytes in both prehypertrophic stage and during osteoarthritis might be related to the necessity of maintaining suffering chondrocytes in a survival state as long as possible. Indeed in both conditions, it has been noticed that the level of ATP was down regulated [44,45]. Considering the vital role of chondrocytes impairment secondary to mitochondrial dysfunction in the initiation and progression of OA, and the fact that gal-3 can prevent the mitochondrial damage and cytochrome c release [46], this could explain in part the protective role of intracellular gal-3 in chondrocyte survival. Consistently with these findings, gal-3 KO mice, have a higher cartilage degenerative score than WT mice [47].

4.2. Extracellular galectin-3 and chondrocyte data

At different concentrations, extracellular gal-3 was able to induce MMP3 and ADAMTS5 expression in chondrocytes, which are two main enzymes involved in proteoglycan degradation in cartilage. When gal-3 was injected into mice knee joints, swelling and OA-like lesions occurred [30]. Two of known gal-3 ligands, fibronectin and integrin subunits are also increased in OA cartilage and one can hypothesize that their recognition by gal-3 may trigger the abnormal metabolism of chondrocytes. The capacity of chondrocytes to bind gal-3 was also verified on human samples. Indeed, Toegel et al. reported recently, that the glycophenotype of OA chondrocytes is not only altered *in vitro* but also *in situ* with progressing cartilage degeneration, in which galectins are present to translate the sugar code modifications into biological functions [48]. They showed that gal-3 binds preferentially to chondrons and

pannus-like tissue, while galectin-1 takes inter-territorial matrix of cartilage and blood vessels as main binding sites [48].

5. Pathophysiological role of galectin-3 in subchondral bone remodeling

The subchondral bone (SCB) plate is the plate underneath cartilage. The line separating calcified cartilage from non-calcified cartilage, called tidemark, is crossed by type II collagen [49], while cement line is the border between cartilage and SCB. SCB is particularly highly vascularized. The high rates of bone blood flow are associated with increased rates of bone remodeling. The architecture of SCB adapts to the mechanical alterations transmitted into the joint, such as elevated thickness, vascularity and perforations, which are more distributed in greater stress regions [49]. SCB is specialized in load bearing, as it attenuates loadings with an efficiency of about 10 times better than cartilage. The deep layer of non-calcified cartilage, the tidemark, calcified cartilage, the cement line and SCB constructed an integrated complex called osteochondral junction. Though the complex is vital for normal joint function, it also provides the convenience to disease progression such as OA. During OA, upward migration of SCB, bone sclerosis, osteophyte formation, SCB cysts and bone marrow oedema-like lesions characterize the SCB alterations [50]. In addition, the increased vascularization and the development of microcracks in the bone matrix reinforce the interaction between cartilage and SCB, as more penetrating channels allow for the exchange areas [51]. On one hand, secreted factors by one of these tissues will follow these channels and modify the biological behavior of the other. For instance, IGF-1 and TGF-β, two growth factors for SCB, are secreted in higher amounts by deteriorated

cartilage, and will partly account for the bone sclerosis. On the other hand, the degradation of cartilage exposes SCB to more loading pressure. Furthermore, the capacity of SCB to respond to mechanical stress reduces due to its alterations in OA, which will in turn make the articular cartilage to suffer from increased loading pressure leading to more severe degradation.

5.1. Galectin-3 and osteoblast data

Expression of gal-3 in osteoblastic cells has been proved and confirmed by a variety of studies [52,53]. Runx2, a transcription factor with a key role in skeletal development, stimulates gal-3 expression in a preosteoblastic murine cell line [53]. Interestingly, dynamic mechanical loading leads to the induction of bone density and strength, and promotes osteoblast proliferation and differentiation, as well as matrix production by activating Runx2. This suggests that intracellular gal-3 may play an important role in the osteoblast physiology.

It was recently reported that gal-3 inhibits human osteoblast differentiation through its binding to Notch 1, which leads to the activation of Notch signaling [54]. Gal-3 was also identified as one of the two principal receptors of advanced glycation end products (AGEs). Mercer *et al* have demonstrated that AGEs induce the expression and secretion of gal-3 in osteoblast-like cells, accompanied with cell apoptosis [55]. The apoptosis may indirectly proceed by AGEs through increasing oxidative stress, or via stimulating pro-apoptotic cytokines expression. However, since the accumulation of AGEs, which increases stiffness of collagen network in bone, is a characteristic of the tissue in aged people, their binding to gal-3 probably have significant consequences on

osteoblast metabolism and thus, on bone turnover [55]. Finally, gal-3 injection into the knee joint has been demonstrated to play a deleterious role on osteoblasts metabolism, as it inhibits osteoblastic markers expression such as osteocalcin, which could explain the abnormal mineralization during inflammatory phases of OA.

5.2. Galectin-3 and osteoclast data

Two early studies showed that preosteoclasts express gal-3 [56,57]. In addition Niida et al demonstrated with electron microscopy, that gal-3 is distributed on the plasma membrane of preosteoclasts, as well as in their cytoplasm and periphery of nucleus and in the extracellular matrix between the cells and bone indicating that gal-3 is secreted [57]. Using an adjuvant-induced arthritis rat model, which is accompanied with severe bone destruction, Li *et al* demonstrated that gal-3 suppresses significantly bone destruction and osteoclast recruitment [58]. They also showed that *in vitro*, extracellular gal-3 inhibits osteoclastogenesis and reduces the dentine resorption by mature osteoclasts. The full length gal-3 was also previously identified as an inhibitor of osteoclastogenesis in MMP9 deficient mice. Indeed this metalloproteinase cleaves extracellular gal-3 in the collagen like domain, which prevents its multimerization and removes the osteoclastogenesis inhibition [59]. More recently, osteolytic metastases of breast cancer were correlated to the presence of MMP13 [60], another metalloproteinase able to cleave gal-3 [42]. They demonstrated that MMP13 and other MMPs and potentially MMP9 promote osteoclastogenesis by cleaving gal-3 [60]. The presence of gal-3 in inflammation could lead to more serious swelling and bone remodeling in mice knee joints [30].

6. Conclusion and perspectives

In summary, arthritis is not a simple process concerning one structure of the joint, but a disease that affects the whole joint. In pathological conditions, synovial tissue, cartilage and SCB are in contact with elevated levels of gal-3, which may play a causative role in the initiation and/or progression of the diseases. Though some progresses have been made in the past decades, the definite role of gal-3 in arthritis has unfortunately not been fully clarified yet. Further *in vitro* or *in vivo* studies will be beneficial to explore its pathological role and may offer us the means, or at least some avenues to slow or even to block gal-3-dependent rheumatic diseases.

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Legends of figures

Figure 1: The structure and classification of different members of the galectin family. (adapted from Blidner AG et al. 2013 and Lepur A et al. 2012)

Figure 2: The details of galectin-3 secretion. To be secreted, galectin-3 accumulates in the cytoplasm underlying plasma membrane domains. These aggregates evaginate and merge with the plasma membrane to form a protrusion that pinch off to release extracellular vesicles from which soluble lectin is released. (Adapted from Hughes RC et al. 1999)

Figure 1

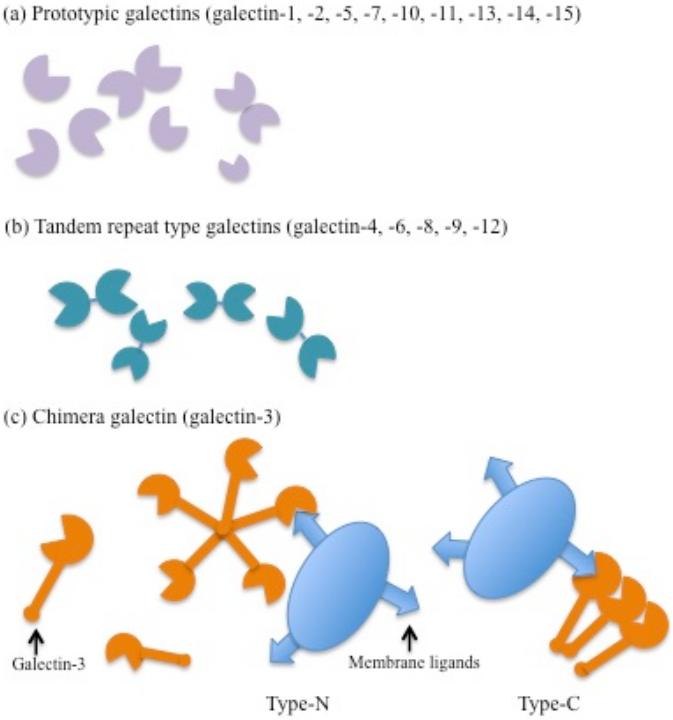
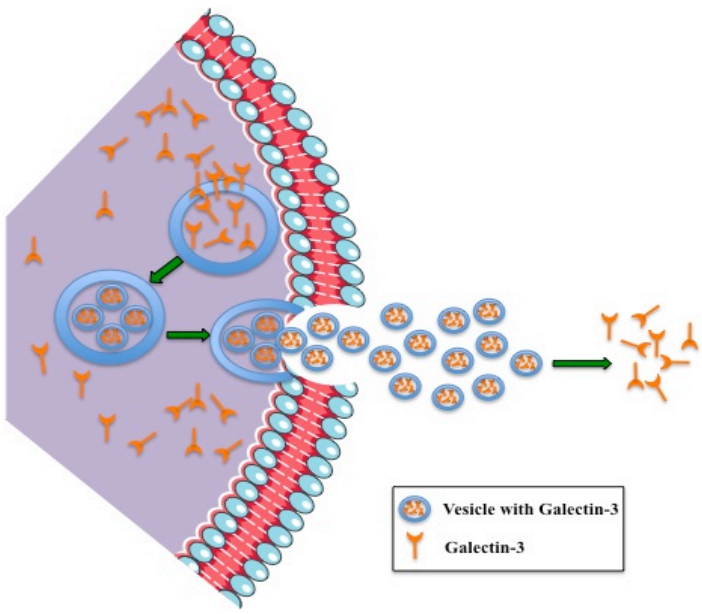


Figure 2



Objectives of our study

Objectives of our study

Cells in SCB are mainly represented by osteoblasts, whose phenotype modifications contribute to the progression of OA. Previously, according to the production of PGE_2, two groups of OA patients have been proposed, namely 'high' and 'low' PGE_2 producers[423]. These authors also demonstrated that the elevated endogenous PGE_2 level is a key modulator of IL-6 production in OA osteoblasts. It is noteworthy that both PGE_2 and IL-6 are involved in bone resorption. However, in late stage of OA, SCB is not osteoporotic but rather presents a sclerosis although the mineralization was abnormal. Therefore, it is necessary to investigate whether other factors modify the phenotype of OA osteoblasts, particularly under Vitamin D_3 stimulation, which is in human being a major control of the late osteoblast differentiation. Previous studies have also shown that OA osteoblasts express higher levels of TGF-β1 and molecules involved in the Wnt pathway [286,423].

Therefore, as first aim we have studied the responses of OA osteoblasts to $1,25(OH)_2$ $VitD_3$ linked to these bone anabolic proteins.

OA symptoms are also associated with inflammatory phases. Galectin-3 (Gal-3) is a pro-inflammatory factor which has been detected in the OA synovial tissue. Combined with cytokines such as TNF-α and IL-1β, Gal-3 is proposed to be responsible for the initiation and/or progression of OA. A detrimental effect of Gal-3 on the cartilage has been found in mice. Concurrently, Janel-Montcalm *et al*. reported that, *in vitro*, Gal-3 stimulates the expression of MMP 3 and ADAMTS-5 in human chondrocytes which are main enzymes responsible for the proteoglycan degradation of cartilage. In addition, SCB histological score also increases in the presence of Gal-3 in mice. Furthermore, without $VitD_3$, the expression of osteocalcin and type I collagen α1 chain is inhibited by Gal-3, especially at its high concentration (10 μg/ml). Osteocalcin is a marker of osteoblast while the latter is the main collagen component of

SCB. The inhibition of these two genes by Gal-3 suggests its role in bone destruction during OA.

Though a few researches related to the effect of Gal-3 in OA cartilage have been conducted, rare reports concerning the relation between Gal-3 and OA SCB alterations are available. Studies conducted in the laboratory have recently demonstrated that the osteoblast phenotype also can be disturbed by different oxygen tension (normoxia and hypoxia)[424]. Therefore, the other parts of this thesis will deal with Gal-3 and OA osteoblasts under both normoxia and hypoxia.

The main objectives are to investigate the modulation of the osteoblast phenotype by Gal-3 and identify the cellular mechanisms involved.

CHAPTER II

CHARACTERIZATION OF OA OSTEOBLASTS

Identification of two populations of osteoarthritic osteoblasts according to the 1,25[OH]$_2$ Vitamin D$_3$ potency to stimulate osteocalcin

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Résumé

Introduction : Jusqu'à présent peu d'études ont essayé de comparer des populations d'ostéoblastes (Obs) arthrosiques (OA). En discriminant deux populations Obs OA sur la base de la production d'ostéocalcine (OCN), nous avons investigué l'expression différentielle de diverses molécules impliquées dans la voie de signalisation Wnt/β caténine.

Méthodes : Des Obs humains OA en premier passage (n = 11) sont incubés en présence ou non de 1,25 dihydroxyvitamine D_3 ($VitD_3$, 50 nM) pendant 24 h. L'expression d'OCN, du facteur de croissance transformant 1 (TGF-β1), de la protéine 2 apparentée au « Dickkopf » (Dickkopf -related protein 2, DKK2), de la R-spondine 2 (Rspo2), de Wnt5b et du récepteur 1 apparenté à celui des LDL (low-density lipoprotein related-receptor 1, LRP1) a été analysée par PCR quantitative en temps réel.

Résultats : Tous les spécimens ont répondu à la $VitD_3$ puisque l'expression d'OCN est augmentée. Cependant deux populations d'Obs ont été discriminées. D'une part les « forts répondeurs » chez lesquels la stimulation d'OCN est supérieure à 100 fois (moyenne 881 fois, $p<0,01$, n = 5) et d'autre part les « faibles répondeurs » ayant une induction d'OCN en dessous de 100 fois (moyenne 47 fois, $p<0,01$, n = 6). En fait, les « forts répondeurs » sont caractérisés par une faible expression basale d'OCN. En comparant ces deux populations et en absence de $VitD_3$, les « forts répondeurs » expriment plus de TGF-β1 (15 fois, $p<0,001$), de DKK2 (2,5 fois, $p<0,002$) et de Wnt5b (5,5 fois, $p<0,003$), alors qu'aucune différence n'est retrouvée pour Rspo2 et LRP1. La présence de $VitD_3$ accentue ce profil d'expression mais corrige le taux d'expression d'OCN et favorise l'expression des agonistes de la voie Wnt.

Conclusion : Nous avons identifié deux populations d'Obs OA grâce à l'expression d'OCN. Dans les conditions basales, ces deux populations expriment de façon différentielle le TGF-β1, Wnt5b et DKK2, ce qui suggère une différenciation et un phénotype hétérogènes des Obs chez les patients OA.

Abstract:
Introduction: Few studies have tried to discriminate a differential behavior between osteoarthritic (OA) osteoblasts (Obs). Based on osteocalcin level, we aimed, in the present study, to evaluate the capacity of OA Obs for producing molecules of the Wnt/β-catenin signaling pathway.
Methods: Human primary OA Obs (n =11) were exposed or not to 50 nM of 1,25 dihydroxyvitamin D_3 ($VitD_3$) for 24 hours. Osteocalcin (OCN), TGF-β1, Dickkopf-related protein 2 (DKK2), R-spondin 2 (Rspo2), Wnt 5b, and low density lipoprotein related-receptor 1 (LRP1) were evaluated by real time RT-PCR.
Results: All samples responded to $VitD_3$ as validated by the increase in OCN expression. However two populations of Obs were discriminated; one called "high responders" whose OCN stimulation was higher than 100 fold (mean 881 fold, $p<0.01$, n=5) and the second one whose stimulation was inferior to 100 fold (mean 47 fold, $p<0.01$, n=6), namely "low responders". In fact, high responders have a weaker basal expression of OCN. With regards to these two cell populations and in absence of $VitD_3$ challenge, the expression level of TGF-β1 (15 fold, $p<0.001$), DKK2 (2.5 fold, $p<0.002$) and Wnt5b (5.5 fold, $p<0.003$) was higher in "high responders", meanwhile Rspo2 and LRP1 expression was unchanged. VitD3 exacerbated this pattern but corrected OCN expression and favored Wnt agonist expression.
Conclusion: We indentified 2 populations of OA Obs according to the OCN expression under the control of $VitD_3$. In addition under basal conditions, these 2 populations expressed differently TGF-β1, Wnt 5b, DKK2, suggesting a heterogeneous differentiation and phenotype in Obs among OA patients.

Key words: osteoarthritis, osteoblast, Wnt system, Vitamin D_3, osteocalcin

Introduction

Osteoarthritis (OA) is considered as a systemic and heterogeneous disease. OA is the most prevalent form of joint disease and a major cause of pain and disability [1]. The end point of OA is cartilage destruction, which is associated with and/or preceded by subchondral bone alterations [2], and synovial membrane inflammation [3]. In OA, it would appear that the extracellular matrix of both cartilage and subchondral bone are altered [4, 5]. Early-stage OA increases remodelling and bone loss whereas late-stage decreases remodelling and increases subchondral bone densification. Both stages are important components of the pathogenic process that leads to OA lesions [6].

In regard to subchondral bone changes, modifications in the production of some molecules synthesized by OA Obs have been shown, i.e. higher levels of osteocalcin and alkaline phosphatase [7, 8], meanwhile the coordination of the alpha1 and alpha2 chains of type I collagen was disrupted [9, 10] resulting in an abnormal mineralization [6, 10]. Among several factors potentially involved in OA pathogenesis, transforming growth factor-β1 [10, 11] and molecules belonging to the Wnt system have also been described [12-14]. It has been reported that in Obs, the Wnt/β-catenin signaling pathway favors the mineralization, which is decreased during OA. This could be linked to the augmentation of TGF-β1, which enhances the expression of DKK2, an antagonist of the Wnt/β-catenin signaling pathway. The alteration of TGF-β1 and DKK2 was, at least in part, responsible for the abnormal osteoblastic phenotype during osteoarthritis [13]. In addition, R-spondin2 (Rspo2), that belongs to a family of four proteins unrelated to other Wnt ligands and acts as a Wnt agonist, is suppressed in OA Obs [14].

Numerous studies have compared the behavior of normal Obs versus OA Obs with the aim of identifying main changes between these two populations of cells. However, OA is a heterogeneous disease and a few studies have tried to discriminate a differential behavior between OA Obs [15, 16]. Based on osteocalcin (OCN), which is a late osteoblastic marker, we aimed, in the present study, to evaluate the capacity of OA Obs for producing molecules which intervene in the Wnt/β-catenin signaling pathway during OA pathogenesis.

Materials and methods

Specimen collection

Human Obs isolated from subchondral bone (tibia plateaus) were obtained from 11 OA patients (F/M: 8/3; aged 68 ± 7, mean ± SD) who had undergone total knee arthroplasty. All patients with OA were verified by certified rheumatologists according to the recognized clinical criteria of the American College of Rheumatology [17]. This procedure was approved by the Ethics Committee of the University Hospital of Nancy.

Subchondral bone osteoblast culture

The overlying cartilage was removed from the tibial plateaus, and then the trabecular bone was eliminated from the subchondral bone plate. The preparation of Obs were performed as described previously [18]. Briefly, the subchondral bone plate was cut into small pieces of 2 mm^2, followed by three successive enzymatic digestions in the presence of 1mg/ml de collagenase type I (Sigma-Aldrich, Saint-Quentin Fallavier, France) without serum in Dulbecco's Modified Eagle Medium (DMEM) at 37°C for 30 min, 30 min and 240 min. After intensive washes in Dulbecco's Phosphate Buffered Saline, the digested pieces were cultured in DMEM/Nutrient Mixture F12 (Gibco®, Carlsbad, CA) containing 10% Foetal Bovine Serum and an antibiotic mixture (100 units/ml penicillin base, 100 µg/ml streptomycin base. Medium was changed every two days. When the cells reached confluency, they were subcultured once at 25,000 cells/cm^2 and grown for 5 days. Confluent cells then were starved for 24h in DMEM/F12 containing 0.5% FBS and then incubated or not in the presence of 50nM $VitD_3$ (Sigma-Aldrich, Saint-Quentin Fallavier, France) for 24h. Ethanol served as vehicle (ethanol 96%, 1µl/ml of medium).

Real time RT-PCR

Total RNA was extracted from Obs using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Gene expression was analyzed by quantitative real-time PCR (Step One Plus Real Time PCR System, Applied Biosystems) using iTaq Universal SYBR Green Supermix (Biorad, Marnes la Coquette, France) according to the manufacturer's protocol. The gene-specific primer pairs are given in table 1. RP29 served as a housekeeping gene.

Table 1. Oligonucleotides used for real time RT-PCR

Gene	Sequences 5′ -3′	Gene ID
RP29	S: GGGTCACCAGCAGCTGTACT AS: CCGATATCCTCCGCGTACTG	NM_001032.3
OCN	S: CATGAGAGCCCTCACA AS: AGAGCGACACCCTAGAC	NM_199173.4
Rspo2	S: CTCCTTTGCCCTCATCATTC AS: TGCAGGCACTCTCCATACTG	NM_178565.4
DKK2	S: GGGTTTTGCTGTGCTCGT AS: TGGCTTTGGAGGAGTAGGTG	NM_014421.2
Wnt 5b	S: AACGTGGAGTATGGCTACCG AS: TGATCCCTTGGCAAAGTTCT	NM_032642.2
TGF-β1	S: GCGTGCTAATGGTGGAAAC AS: GCTGAGGTATCGCCAGGAA	NM_000660.5
LRP1	S: AGCAGTTTGCCTGCAGAGAT AS: CTGGGCCTTACTCTGTGGAC	NM_002332.2

Statistical analysis

Data are expressed as mean ± SD. Statistical analyses were performed using the two-tailed Student's t tests versus unstimulated osteoblasts (ctrl) or versus "low responders". Results of $p<0.05$ were considered significant.

Results

1. Discrimination of two populations of OA osteoblasts according to osteocalcin expression

The increase in OCN expression, a late marker of osteoblast differentiation, under 1,25 dihydroxyvitamin D3 ($VitD_3$) stimulation indicated that all cell cultures corresponded to Obs (figure 1A). However, according to the scattered data, we considered separating our samples in two populations; one so-called "High responders" whose mean induction of OCN was >100-fold (mean +/- SD: 880-fold +/- 94) (figure 1B, n=5) and another one namely "Low responders" whose OCN was <100-fold (mean +/- SD: 47-fold +/- 3) (figure 1C, n=6) versus non stimulated cells (Ctrl).

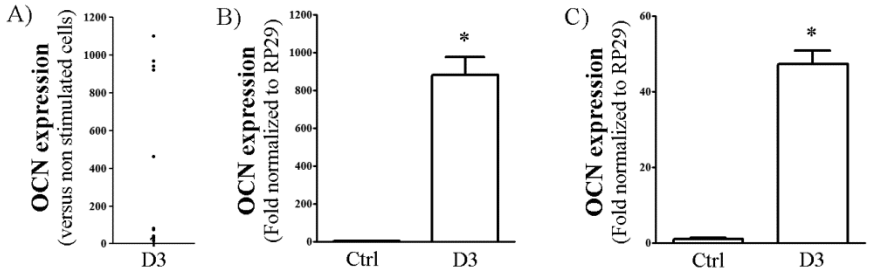

Figure 1. VitD_3-induced OCN expression in OA osteoblasts. A) scatter plot of OCN expression coming from 11 OA samples. B) OCN expression (mean ± SD) in "high responders". C) OCN expression (mean ± SD) in "low responders". Threshold between high and low responders was arbitrary given for an induction above or below 100 fold, respectively. * = $p<0.05$ versus Ctrl

In fact, by analyzing results in absence of VitD_3 in each population we found that basal expression of OCN in Low responders was twenty times higher than in High responders (1 ± 0.46 *vs* 0.05 ± 0.03, respectively; $p<0.05$). In other words, Low responders had initially a high level of basal OCN expression whereas High responders had a lower level of expression, whereas VitD_3 promoted same final amount of OCN expression in both populations.

2. Expression of TGF-β1, Wnt5b, DKK2, Rspo2 and LRP1 in these two populations

As TGF-β1 and several members of the Wnt family have been considered to be involved in the modification of the OA osteoblastic phenotype, we evaluated whether these genes were differently expressed in these two cell populations.

The most spectacular differences were found for the TGF-β1 and Wnt5b basal expressions with an increase of 15-fold for TGF-β1 and 5.6-fold for Wnt5b in the High responder population compared to the Low responder group (figure 2A, 2B; $p<0.05$). High responders also expressed 2.5 times more DKK2 (figure 2C, $p<0.05$). Conversely, Rspo2 expression tended to be lower in High responders (inhibition of 30%) although no significance was reached (figure 1D) meanwhile LRP1 expression was similar in the two cell populations (data not shown).

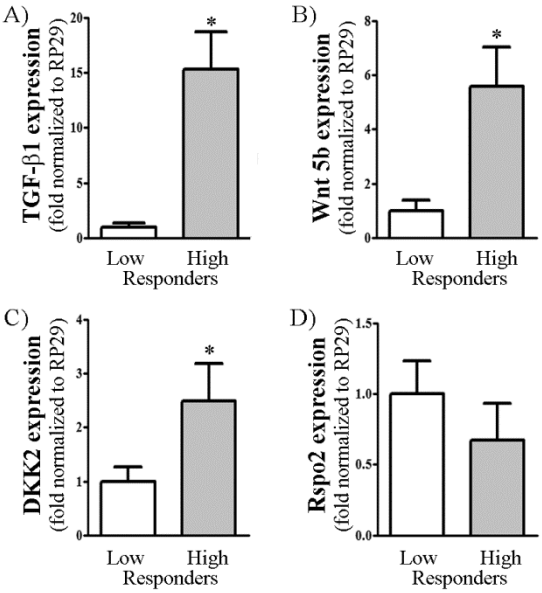

Figure 2. Basal expression in the Low and High responder population of several genes, known to be involved in OA pathogenesis.

The low responders served as the calibrator for transformation of ΔΔCt into fold expression.
Bars represent mean ± SD.
Low responders n = 6.
High responders n= 5.
* = $p<0.05$ versus low responders

3. Expression of TGF-β1, Wnt5b, DKK2, Rspo2 and LRP1 under $VitD_3$ stimulation in these two populations

As $VitD_3$ deficiency has been suggested to play a potential role in OA, we have incubated Obs with this vitamin in order to verify if we could modulate differently the expression of the genes cited above in these two osteoblastic populations. These two populations did not exactly respond to $VitD_3$ in the same way for a particular gene. For instance, TGF-β1 expression was down-regulated by $VitD_3$ in the Low responders whereas the cells from patients in the High responders group seemed to escape this regulation (figure 3A) which led to a shift from 15-fold to 20-fold of TGF-β1 expression between low and high responders in basal versus $VitD_3$-stimulated conditions. Conversely, Wnt5b expression was enhanced in High responders in response to VitD3 meanwhile it was unchanged in Low responders (figure 3B) triggering an enhancement of Wnt5b expression by 1.4-fold between these two populations. Finally, DKK2 and Rspo2 were regulated in a similar manner, in which $VitD_3$ induction of each gene was more pronounced in High responders than in Low responders (figure 3C, 3D) increasing by 1.3- and 2-fold, respectively, the expression of these genes in the two populations. Moreover, the Wnt5b/DKK2 expression ratio varied from a relative value of 1 for the Low responders to 2.5 for the High responders. Last, LRP1 expression was not modulated by VitD3 in the two cell populations (data not shown).

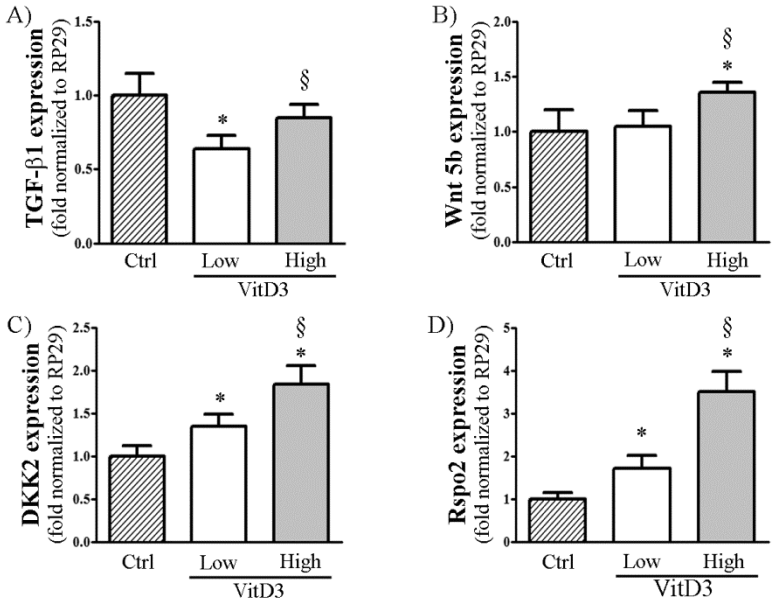

Figure 3. $VitD_3$-induced expression in the Low and High responder population of several genes, known to be involved in OA pathogenesis. As Ctrl (ethanol = vehicle) served of calibrator for transformation of ΔΔCt into fold expression, (value =1) for each population only one "Ctrl" histogram is shown. However calculations were made with the right values for each group. * = $p<0.05$ versus Ctrl; § = $p<0.05$ versus Low

Discussion

During OA, subchondral bone sclerosis indicates that an increase of osteogenesis occurs, yet OA bone is hypomineralized. Mechanisms of this hypomineralization are not well defined but abnormal osteoblastic differentiation has been described for OA bone. Indeed some findings indicate that TGF-β1 and factors involved in the Wnt signaling pathway may be involved in this hypomineralization. Therefore our study aimed to evaluate the expression of these factors in relationship with the differentiation of OA Obs.

First of all we confirmed that cells cultured in our experimental conditions were Obs. For that goal, we have incubated our cells with $VitD_3$, whose properties are well known for stimulating specific expression of osteoblastic markers such as osteocalcin [19]. As every specimen did respond to $VitD_3$ challenge, we were confident we were working with Obs. However, the intensity of osteocalcin expression in response to $VitD_3$ was

variable according to specimens, which have permitted to discriminate these samples in two populations. In fact the basal expression of osteocalcin in these two populations was the cause of the fluctuation of osteoblastic sensitivity to $VitD_3$. We have identified High and Low responders to $vitD_3$, High responders having low basal level of osteocalcin expression whereas, Low responders had initially high osteocalcin expression levels. Differential basal level of osteocalcin has been attributed to a more or less differentiated osteoblastic state [20], the most differentiated Obs having the highest level of osteocalcin expression. These findings suggest that the differentiation state of Obs is not the same from one OA patient to another but should be confirmed in a larger group of OA patients.

The less advanced state of differentiation of Obs coming from the High responders could be explained by their potential to express higher Wnt 5b levels and may be other agonists of the Wnt pathway [20]. This hypothesis could be especially valuable since despite the increase in DKK2 expression (antagonist of Wnt pathway) in the High responders group, the Wnt 5b/DKK2 ratio varies from a relative value of 1 for the Low responders to 2.5 for the High responders, favoring therefore, a potential local response to Wnt 5b. This latter could inhibit late osteoblastic differentiation. It would be also interesting to compare or correlate the decrease of Rspo2 expression, although not significant, in the High responders with the "High-PGE_2 responsive OA" Obs identified previously by the production of PGE_2 and IL-6 [14, 16]. Indeed although these previous works did not analyze the expression of Rspo2 between their identified groups, it seems that the "High-PGE_2 responsive OA" cells expressed less Rspo2 than the "Low-PGE_2 responsive OA"ones [14]. In addition the same work demonstrated that the addition of TGF-β1 decreased Rspo2 production. Yet, it turns that the trend of Rspo2 decrease could be associated to the high level of TGF-β1 in our High responder group. In addition, another study has shown that TGF-β1 inhibited the late maturation of Obs [21]

One hypothesis for OA progression is that factors coming from subchondral bone reach deep layers of cartilage and change the chondrocyte phenotype. For instance, a duplication of the tide mark is found in OA cartilage as well as hypertrophic markers. Some findings showed that Wnt 5a and Wnt 5b inhibited the hypertrophic differentiation of chondrocytes [22]. Therefore, it seems that the amount of Wnt 5b produced by OA Obs would not be sufficient to prevent chondrocyte phenotype changes. However, we have also demonstrated that Wnt 5a was responsible for the loss of articular chondrocyte phenotype in the presence of IL-1 [23]. Thus, it could happen that in OA cartilage the mixture of several factors may trigger Wnt 5b to be involved in the phenotype change of chondrocytes. Indeed, it has been suggested that Wnt5a or Wnt5b may modulate production of proliferative zone chondrocytes and their conversion into hypertrophic chondrocytes [24]. Thus, as for Obs, chondrocytes could express a number of several altered phenotypes in OA articular cartilage.

As mentioned above, the Wnt pathway depends on both the presence of Wnt receptors and cofactors. For instance Wnt 5a was previously shown to only activate the non-canonical route of Wnts. However, we and others have demonstrated that this factor activated the canonical pathway as well [23, 25]. This latter pathway activation was induced in the presence of LRP1 [25]. Moreover, LRP1 is an important receptor for vitamin K internalization in Obs. This vitamin is an essential factor for the gamma carboxylation of glutamic residues of osteocalcin [26], which then plays fully its role in the mineralization process. However, as LRP1 was not differently express in the Low and High responders either in basal or $VitD_3$ conditions, this factor seems not to be crucial in the OA process.

Conclusion

We have clearly identified two populations OA Obs. These Obs are in differential stages of differentiation in an OA patient-dependent manner suggesting possibly variable developmental stages of the disease.

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CHAPTER III

MODIFICATION OF COLLAGENASE 1 AND LEPTIN EXPRESSION BY GAL-3 UNDER DIFFERENT OXYGEN TENSIONS

Materials and Methods (supplementary data 1)

3.1 Purification of Galectin-3 (All buffers are referred in annex 1)

3.1.1 Bacteria culture

Pick a single colony (bacteria BL21 C41 which has been transformed with PTrc his-Gal-3) and culture in 3 ml LB with 6 µl ampicillin (final concentration 100 µg/ml) at 37°C with shaking for 4-6 hours. Then mix this volume to 150 ml Luria Bertani medium (LB) + 300 µl ampicillin at 37°C with shaking for overnight. The following morning, 25 ml of suspension were dispatched in 500 ml LB + 1 ml ampicillin (final concentration 100 µg/ml) at 37°C with shaking. When the absorbance of bacterial suspension reaches 0.6 to 0.8, IPTG was added at a final concentration of 1 mM. Then the bacteria were cultured at 20°C with shaking for overnight. Once bacteria culture was harvested, the suspension was centrifuged at 4°C, 6000 rpm, 5-10 minutes in order to obtain the bacteria pellet, which was frozen at -80°C.

3.1.2 Sonication of bacterial suspensions

The pellet was thawed and suspended in 40 ml cold buffer B. Then the bacteria suspension was disrupted by sonication (4 kHz) for 1 min and vortexed for another 1 min. This step was repeated 3 times in an ice bath. To achieve disruption, the suspension was frozen at -80°C again. This overall procedure was repeated for 3 times.

3.1.3 Purification on lactose agarose column

The suspension was thawed before adding 3.2 g of NaCl. Then the mixture was vortexed 2×2 min and the volume was adjusted to 400 ml with buffer B. After a centrifugation at 8000 rpm for 45 min, the supernatant was recovered while the pellet discarded. Then, the supernatant was loaded onto a column of lactose agarose which was beforehand equilibrated with the equilibrating buffer. The column was rinsed with buffer C and PBS. Finally, the column was eluted with 25 ml buffer PBS containing lactose at 150 mM. Samples are picked after every step described above for SDS PAGE analysis.

3.1.4 Purification on probond column

The eluate from lactose agarose was mixed with a probond© resin, equilibrated with PBS, in a 50 ml tube. The tube was agitated overnight on the rotor at 4°C. The following day, the probond© resin was decanted in a chromatographic column, rinsed with PBS at pH: 8 and eluted with 15 ml of the same buffer containing imidazole at 250 mM. As for lactose agarose column, samples were taken after every step for SDS PAGE analysis.

3.1.5 Dialysis

A dialysis membrane with a cut off at 10 kDa was rinsed into distilled water and clipped at one end. After having verified that the membrane was not leaking, it was filled with the eluate and clipped at the other end. The membrane was fixed on the marge of one beaker which has been filled with PBS under shaking at 4°C for 24 h. PBS was changed at least 3 times. At the end of the dialysis, the eluate was transferred into a falcon© tube.

3.1.6 Endotoxin elimination

The experiment was executed under a cell culture hood. The post-dialysis eluate was cleared off of endotoxins with a 5 ml E-TOX column© and conserved at -80°C.

3.1.7 Protein lyophilization and Gal-3 quantity measurement

After lyophilization, Gal-3 was solubilized with Dulbecco's Phosphate-Buffered Saline (DPBS), and the concentration was estimated by absorbance at 280 nm or by bicinchoninic acid (BCA) method. The concentration pre- and post- lyophilization was measured to evaluate the loss of protein. For spectrometric estimation of Gal-3, the nanodrop was set at the molecular weight 27.65 (kDa) and ε 33.83 (x1000).

Method of BCA

The commercial BSA solution (1 mg/ml) was used to make standard curve. Calibration scale of BSA was made in duplicates for amounts ranging from 0 to 20 µg in a final volume of 60 µl of DPBS. Estimation of Gal-3 was performed with volumes ranging from 10 to 60 µl. The reagent is composed of BCA solution and Cu_2SO_4 4% in a

proportion of 50:1, was dispatched in a volume of 240 µl both in standards and samples. After an incubation of 30 min at 37°C, the absorbance was measured at 562 nm.

3.1.8 Electrophoresis of the samples

In order to evaluate the quality of the purification, aliquots which have been taken out during the purification were analyzed on SDS-PAGE. Aliquots were mixed vol/vol with a 2x concentrated Laemmli's buffer, incubated at 95°C for 5 minutes and cooled immediately. They were migrated at 100 V for at least 1 hour in an acrylamide gel of gradient concentration 4 to 20%. At the end of migration, the gel was fixed in the fixative solution for overnight.

3.1.9 Silver staining

The gel was washed 2 times with ultrapure water for 30 min each, immersed in the sensitizer for 1 min, and then impregnated with a solution of silver nitrate. After 2 quick washes in water, the gel was developed until proteins bands appeared (Figure 3.1). Development was stopped for 30 min.

3.1.10 Analysis of nitrites (Griess reaction)

Human chondrocytes were incubated with the purified Gal-3 (5 µg/ml), IL-1 (1 ng/ml, positive control) and PBS (negative control) for 24 h. Culture supernatants were collected to analyze the nitrites, and cells were lysed with 0.5% of SDS for protein estimation by BCA.

Nitrite standard curve was realized with a solution of 10 mM $NaNO_2$ (final concentration ranges from 0 to 50 µM). Reagent solution was a mixture vol/vol of naphthylethylenediamine dihydrocholoride 0.1% in water and sulfanilamide 1% in 5% H_3PO_4. Nitrite was estimated in 100 µl of culture supernatant. The final volume of the reaction was 200 µl. After 10 min, the absorbance was read at 550 nm. Nitrite measurement informed us indirectly whether Gal-3 was contaminated by endotoxins.

Annex 1

buffer A	Tris 20 mM, EDTA 5 mM, adjust up to 1L with ultrapure water, pH 7.5.
buffer B	buffer A 500 ml, DTT 1 mM, 10 tablets of proteinase inhibitors.
equilibrating buffer	buffer A 500 ml, NaCl 150 mM.
buffer C	equilibrating buffer 100 ml, DTT 1 mM.
buffer D (PBS)	NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.78 g, KH_2PO_4 0.24 g, adjust up to 1L with ultrapure water, pH 7.4.
buffer E	buffer PBS 25 ml , lactose 150 mM.
buffer PBS pH 8	NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.63 g, NaH_2PO_4 0.11 g, adjust up to 1L with ultrapure water, pH 8.
buffer PBS pH 8 imidazole 250mM	buffer PBS pH 8 50 ml, imidazole 250 mM.
Fixative solution	Methanol 300 ml, acetic acid 100% 100 ml, distilled water 600 ml.
Sensitizer	$Na_2S_2O_3$ 100% 200 mg, distilled water 1000 ml.
Impregnation buffer	$AgNO_3$ 1 g, formaldehyde 37% 2 ml, distilled water 998 ml.
Developer	K_2CO_3 30 g, $Na_2S_2O_3$ 100% 12.5 mg, formaldehyde 37% 675 µl, distilled water 1000 ml.
Stop solution	Acetic acid 100% 10 ml, distilled water 990 ml.

3.2 Identification of galectin-3 ligands **(All buffers are referred in annex 2)**

3.2.1 Immobilization of galectin-3 on NHS-activated Sepharose

For coupling galectin-3 onto NHS-activated Sepharose, the elution from the lactose agarose column was performed with the coupling buffer containing 150 mM of lactose. The blocked NHS-activated Sepharose solution was homogenized and poured into a 50 ml tube in order to get 5 ml resin. The resin was then rinsed twice with 30 ml of

ultrapure water and activated with 35 ml HCl 1 mM. The purified galectin-3 and the activated resin were mixed, left at 4°C under rotation for overnight. The next day, the resin was settled and the supernatant discarded. The coupling reaction was stopped with 35 ml of buffer 1 for 4 h at 4°C. Then the galectin-3 Sepharose resin was washed three times with 35 ml of buffer 2 and at last twice with 35 ml of buffer 3. After settling and aspiration of buffer 4, the galectin-3 Sepharose resin will be stored at 4°C in 10 of ml buffer 4, until use. In order to verify that galectin-3 was coupled to NHS-activated Sepharose resin, aliquots were taken after every step of the resin preparation and were analyzed by SDS-PAGE. Gels were stained with Coomassie brilliant blue (Figure 3.2).

3.2.2 Extraction of membrane-enriched fractions from human OA osteoblasts

Once osteoblasts were confluents in the flask, they were trypsinized and centrifuged at 1500 rpm for 5 minutes. The pellet was rinsed twice with PBS and stored dry at -80°C. The pellets obtained from 15 specimens were pooled, homogenized in 500 µl of buffer 5 (annex 2), sonicated (4 kHz) three times for 10 seconds in an ice bath and centrifuged at 10,000g for 10 minutes. Then the supernatant was recovered and centrifuged at 100,000 g for 1 hour. The pellet containing the membrane-enriched fractions was solubilized and carefully homogenized in buffer 6. The suspension was centrifuged at 10,000 g for 10 min to get rid of the membrane debris and the supernatant was collected for evaluating potential Gal-3 ligands.

3.2.3 Screening of potentials ligands in Gal-3 Sepharose

Experiment procedures are described in the article entitled “Screening of potentials ligands in Gal-3 sepharose” and raw results obtained after the elution of Gal-3 Sepharose column are shown in Figure 3.3.

3.2.4 Ligands identification on western blot

Gal-3 coupled to digoxenin (Dig) at a concentration of 250 ng/µl was already home made during the work of C. Perrin, who made her L3 laboratory in our group. The flow through and the eluted fractions of Gal-3 Sepharose column were concentrated by acetone at a ratio of 1:3 for overnight at -20°C. After centrifugation at 12000 rpm for 15 min, the pellets were suspended in 1× Laemmli buffer. Two equal parts of each fraction

were separated by SDS-PAGE and transferred onto PVDF membrane. This membrane was blocked for 24 hours with the blocking solution consisted of blocking reagent (Ref 11096176001, Roche, Germany) diluted in Tris-buffered saline (TBS, referred in annex 2) at a concentration of 0.5%. Then, the membrane was cut in 2 parts, one incubated with Gal-3-Dig 1 µg/ml diluted in the blocking solution for O/N, the other incubated with the same mix but containing also 300 mM of lactose. After three washes in TBS for 10 minutes, Anti-Dig-POD (peroxidase) Fab fragments were diluted in the blocking solution at a ratio of 1:2000. After three washes in TBS, detection was performed with by ECL substrate (Figure 3.4).

Annex 2

Coupling buffer	$NaHCO_3$ 0.2 M, NaCl 0.5 M, pH 8.3.
buffer 1	Tris-HCl 0.1 M, pH 8.5.
buffer 2	Tris 50 mM, NaCl 0.5 M, pH7.5.
buffer 3	Tris 20 mM, NaCl 150 mM, pH7.5.
buffer 4	(Tris 20 mM, NaCl 150 mM, pH7.5): Ethanol 4:1
buffer 5	Hepes 5 mM (pH 7.4), saccharose 0.25 M.
buffer 6	Hepes 5 mM (pH 7.4), saccharose 0.25 M, triton-x100 0.05%.
TBS	Tris 50 mM, 150 mM NaCl, pH 7.5.

3.3 Dosage of MMP1 and Leptin in cellular medium by ELISA (Ref DY901 and DY398-05, RD, France)

(Other solutions required are referred in annex 3)

3.3.1 Plate preparation

The capture antibody was diluted at the working concentration (MMP1 2 µg/ml, Leptin 2 µg/ml) in PBS without carrier protein. Then, a 96-well microplate was immediately coated with 100 µl per well of the diluted capture antibody, sealed and incubated overnight at room temperature. The next day, each well was aspirated and rinsed with 400 µl of wash buffer using a squirt bottle for a total of three washes. After the last wash, any remaining buffer was removed by inverting and blotting the plate against clean paper towels. The plate was then blocked by adding 300 µl of Reagent Diluent in each

well and was incubated at room temperature for 1 hour. Finally the plate was washed as described above, before being ready for the assay procedure.

3.3.2 Assay Procedure

One hundred µl of sample or standards in Reagent Diluent for a total volume of 100 µl were added to per well. The plate was sealed and incubated 2 hours at room temperature. After a sequence of 3 aspirations and washes, 100 µl of the biotinylated secondary antibody diluted in Reagent Diluent (MMP 1 100 ng/ml, Leptin 25 ng/ml) was added to each well for another incubation of 2 hours at room temperature. The plate was washed as described above, before adding 100 µl of the working dilution of Streptavidin-HRP into each well. After an incubate for 20 min at room temperature and a sequence of 3 aspirations and washes, the coloration was developed with 100 µl of substrate solution for 20 min at room temperature, preferentially in a manner avoiding direct light. The reaction was stopped by adding 50 µl of Stop Solution to each well. Gently tap the plate to ensure thorough mixing. Determine the absorbance of each well immediately, using a microplate reader set at 450 nm.

Annex 3

PBS	Na_2HPO_4 8.1 mM, NaCl 137 mM, KCl 2.7 mM, KH_2PO_4 1.5 mM, pH 7.2-7.4, 0.2 µm filtered.
Reagent diluent	1% BSA in PBS, pH 7.2-7.4, 0.2 µm filtered.
Wash buffer	0.05% Tween® 20 in PBS, pH 7.2-7.4.
Substrate solution	1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine)
Stop solution	2 N H_2SO_4

Results (supplement data 1)

3.1 Purification of Gal-3

During the purification of Gal-3, samples are collected in every step to evaluate the efficiency of purification (Figure 3.1). Electrophoresis of the aliquots showed us that many proteins are present in bacteria lysate (A, B). After pass the lysate supernatant on the column of lactose agarose, Gal-3 binds with the resin, other proteins which can not bind with the resin will be present in flow-through (C). After the elution of the column by lactose, a good quantity of protein mainly with the weight about 27 kDa is obtained (F). Second column with probond resin is used to further purify the protein, compared with (F), the eluted protein after column probond (I) is more pure but less intense, which means the loss of protein during the purification. This was further progressed after the removal of endotoxin by E-TOX column (J).

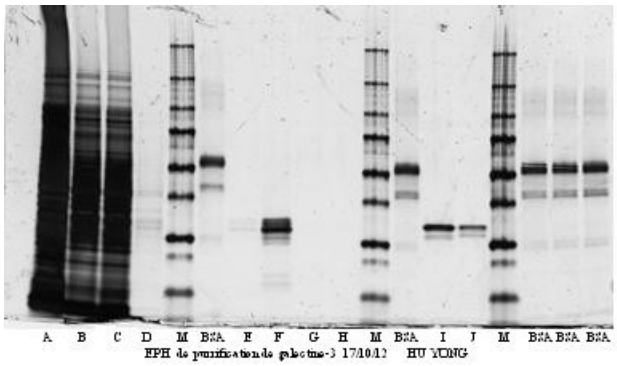

Figure 3. 1: Electrophoresis of the aliquots from the purification of Gal-3. Samples order describe as following procedures: A Bacteria lysates, B Supernatant after the lysate centrifugation, C Flow through after passing the supernatant on the column of lactose agarose, D wash with equilibrating buffer, E wash with buffer PBS, F Eluate from the column of lactose agarose, G Supernatant after binding with the resin of probond column, H wash with buffer PBS pH8, I Eluate from the probond column, J Eluate after pass the E-TOX column, M Marker, BSA bovine serum albumin.

3.2 Identification of Gal-3 ligands

3.2.1 Immobilization of Gal-3 on NHS-activated sepharose

To identify Gal-3 ligands, a big quantity of purified Gal-3 (50 mg) should be coupled to the NHS-activated sepharose (Figure 3.2). Compared the purified Gal-3 before coupling (A) and supernatant after binding (B), we can conclude that large amounts of Gal-3 has been coupled to the resin. Of course, the following steps are also leading to loss of Gal-3 binding.

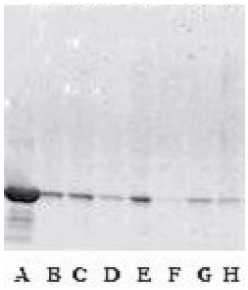

Figure 3. 2: Electrophoresis of the aliquots during immobilization of Gal-3 onto NHS-activated Sepharose. Samples are loaded as following procedures: A The purified Gal-3 before coupling, B Supernatant after binding, C Mixture of Gal-3 and resin after binding, D Supernatant after settling, E Recombinant Gal-3 (positive control), F Flow through after adding the stop solution, G Flow through after the first wash, H Flow through after the second wash.

3.2.2 Screening of potentials ligands in Gal-3 Sepharose

After pass the enriched osteoblast membrane fractions on the column Gal-3 sepharose, the potentials ligands of Gal-3 are eluted by lactose (Figure 3.3). Compared with column BSA sepharose which has been coupled with BSA, the elution of column Gal-3 sepharose contains many proteins, some of which are very intensive (red arrows).

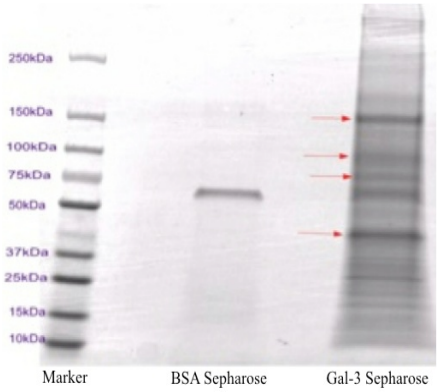

Figure 3. 3: SDS-PAGE of the elution from the column of BSA-Sepharose (negative control) or Gal-3-Sepharose by lactose.

3.2.3 Ligands identification on western-blot

The elution of column Gal-3 sepharose was also analyzed in western-blot based on lectin recognition (Figure 3.4). Left panel show us that in the elution, an intensive band around 150 kDa is present, while nothing is found in the flow-through. Right panel deals with the same condition but in presence of lactose (300 mM), interestingly, the band what we found in elution in left panel is no longer exist. It means that the protein in the band around 150 kDa binds with Gal-3 in a lectin-recognition manner.

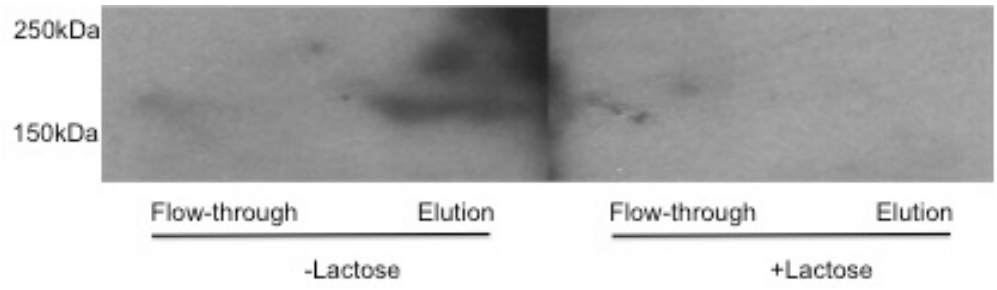

Figure 3. 4: Identification of potential ligands by lectin-recognition. The elution and flow-through of Gal-3 sepharose column was migrated in SDS-PAGE, and two equal membranes were incubated with Dig-Gal-3 in presence of lactose (300 mM, right panel) or not (left panel). Signal was detected by ECL corresponding to a band around 150 kDa (right panel).

3.3 Dosage of MMP 1 and leptin in culture medium by ELISA

(see in article)

Collagenase 1 and leptin are differently regulated by several galectin-3 ligands in human osteoarthritic osteoblasts

Y. Hu, A. Bianchi, J.B. Vincourt, P. Netter, D. Mainard, J-Y. Jouzeau, P. Reboul

Objectifs

La galectine-3 (gal-3) est une lectine capable de se lier les structures β-galactoside qui peut être retrouvée en quantité importante durant les phases inflammatoires notamment au niveau de la membrane synoviale. Le rôle délétère de gal-3 a été suggéré dans l'arthrose (OA). Durant cette maladie il y a aussi des problèmes de circulation osseuse au niveau de l'os sous-chondral, ce qui engendre aussi de l'inflammation et des zones hypoxiques. Ces évènements sont associés à l'expression de MMP 1 et de la leptine. Le but de cette étude est de déterminer si ces 2 gènes sont régulés par gal-3 dans les ostéoblastes OA humains que ce soit en normoxie ou hypoxie et d'explorer les mécanismes cellulaires sous-jacents.

Méthodes

Les ostéoblastes de l'os sous-chondral de plateau tibial ont été cultivés soit sous 21% (normoxie) soit sous 2% d'oxygène en présence de gal-3 (5µg/ml). L'expression de MMP 1 et de la leptine a été mesurée par RT-qPCR. Les voies de signalisation cellulaire actives par gal-3 ont été étudiées par western blotting, puis en utilisant des inhibiteurs correspondant à ces voies, la régulation cellulaire de MMP 1 et de la leptine a été investiguée. Enfin, les ligands à la surface des ostéoblastes capables de médier l'effet de gal-3 ont été identifiés par chromatographie d'affinité sur colonne de gal-3-Sepharose et spectrométrie de masse. Certains ligands ont été choisis and éteints par siRNA pour évaluer leur rôle sur l'expression de MMP 1 et de la leptine.

Résultats

Tel que mesuré par RT-qPCR et ELISA, la gal-3 stimule l'expression de MMP 1 avec un pouvoir similaire que ce soit en normoxie ou hypoxie. La régulation de cette stimulation dépend des voies cellulaires P38 MAPK et PI3K. Parmi plusieurs ligands potentiels identifiés sur chromatographie et spectrométrie de masse, 4F2hc, and CD44 sont les ligands majeurs capables de réguler positivement MMP 1 sous l'influence de gal-3 tandis que l'intégrine β5 régule négativement MMP1 sous un mode gal-3 indépendant. L'expression de la leptine est également stimulée par gal-3 et cette régulation implique les voies cellulaires ERK1/2, P38 MAPK et PI3K. Contrairement à MMP 1, 4F2hc et l'intégrine β5 régulent très fortement la leptine.

Conclusions

Nos résultats démontrent que la gal-3 à la capacité de perturber l'homéostasie osseuse en induisant MMP1 et la leptine. De plus, notre étude montre que la gal-3 régule la leptine et MMP1 en utilisant soit un seul ou plusieurs ligands membranaires et en générant de nombreux signaux intracellulaires. C'est pourquoi, pour contrer les effets de gal-3, il semble plus facile d'empêcher celle-ci de se lier que d'utiliser des inhibiteurs de sa signalisation cellulaire.

Collagenase 1 and leptin are differently regulated by galectin-3 ligands in human osteoarthritic osteoblasts

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ABSTRACT

Galectin-3 (gal-3) is an endogenous β-galactoside-binding lectin, found highly expressed in the inflammatory situation that can be recovered in synovium. The presence of galectin-3 has been demonstrated to play a role in osteoarthritis (OA) initiation and/or progression. During the OA, the venous occlusion and stasis or the development of micro-emboli in the vessels of subchondral bone will trigger ischemia, which may generate a hypoxic condition. These events are associated with bone remodeling involving MMP1 and leptin expression. The aim of this study is to investigate whether these genes are regulated by gal-3 in human OA osteoblasts (Obs) under different oxygen tension and to explore the cellular mechanisms by which gal-3 may act. Gal-3 stimulates the expression of MMP1 to the same extent under normoxia and hypoxia. Gal-3-stimulated MMP1 was strongly regulated by P38 MAPK and PI3K pathways. Among several potential ligands, 4F2hc and integrin β1 are the major ligands able to positively regulate MMP1 by gal-3 while integrin β5 negatively regulates MMP1 in a gal-3 independent pathway. Leptin expression is also stimulated by gal-3 and this regulation involves ERK1/2, P38 MAPK and PI3K pathways. 4F2hc and integrin β5 are positive regulators of gal-3-induced leptin expression. Our results demonstrated that gal-3 has the potential to disturb bone homeostasis by inducing MMP1 and leptin. In addition, our study demonstrates that gal-3 regulates leptin and MMP1 by using either one or several cell surface ligands and by inducing numerous intracellular signaling pathways. Therefore, whether gal-3 has a deleterious role in joint, the easiest way to counteract its effects is to prevent its binding.

Key words: osteoarthritis, osteoblast, collagenase 1, galectin-3, leptin,

Introduction

Osteoarthritis (OA), whichis characterized by a degradation of articular cartilage and abnormal bone remodeling, is the most prevalent form of arthritis and a major cause of pain and disability in elders[1]. The joint degradation is manifested by the depletion of collagens and proteoglycans due to the production of matrix metalloproteinases (MMPs), aggrecanases (ADAMTSs) and other proteases. The production of these degrading enzymes is driven by a host of inflammatory mediators within the affected joint, such as interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-a), IL-6, nitric oxide (NO), prostaglandins and others. Among these factors, galectin-3 (gal-3) may play a role.

Until now, 15 galectins have been identified in a variety of tissues. Galectin family is divided in three different subgroups, namely the prototype, the tandem repeat-type and the chimera type. Gal-3 belongs to the latter and is composed of an evolutionary conserved sequence element of carbohydrate-recognition domain (CRD) which is characterized by specific binding to β-galactosides. In addition to this lectinic domain, gal-3 contains an N-terminal domain, through which it forms oligomers. First discovered in macrophages, gal-3 has been found to be widely distributed in tissues, like brain, kidney, prostate and skeleton[2, 3]. Although gal-3 does not contain a signal peptide, it can be secreted into extracellular compartment in a non-classical pathway which has yet not been deciphered but seems to be related to ectocytosis[4]. It has been reported that gal-3 participates in a variety of biological processes depending on its subcellular localization, such as RNA splicing, differentiation, apoptosis, cell-cell or cell-matrix interactions.

During OA inflammatory phases, activated synovial macrophages secrete gal-3 into the synovial fluid, and hig gal-3 levels have been shown to be deleterious for joint tissues. Thus, MMP3 and ADAMTS5, which are enzymes involved in cartilage degradation, are stimulated by gal-3 in chondrocytes[5]. In addition to cartilage, subchondral bone (SCB) is also a target tissue, as gal-3 down-regulates osteocalcin production in osteoblasts[5]. Interestingly, a recent study has shown that MMP1, an interstitial collagenase that is capable of degrading collagens, is elevated in SCB for which Mankin' grade is high[6]. As it is well known that inflammatory phases aggravate the severity of joint pathological score and that gal-3 is able to induce MMP3 in chondrocyte, it would be attractive to evaluate whether gal-3 could also regulate

MMP1, the main enzyme having the capacity to degrade the osteoid matrix. In addition, our group has recently demonstrated that osteoblasts were particularly sensitive to hypoxia. Indeed under hypoxic conditions they produce high level of leptin[7], which is another factor well known for favoring osteoarthritis[8, 9].

However, the molecular mechanism how gal-3 carries on its effects remains obscure in joint cells. In cells other cell types, ligands of gal-3 have been proposed, such as laminin[10, 11], fibronectin[12], elastin[13], collagen[14], integrin β1[15] and others. A recent report also indicates that prostate specific antigen, prostatic acid phosphatase, zinc alpha-2-glycoprotein, dipeptidyl peptidase-4 (CD26) and aminopeptidase N (CD13) are also gal-3 ligands[16]. Another study suggested that that gal-3 colocalized with N-cadherin at carcinoma cell-cell junction[17].

Therefore, the aims of this study are to investigate the effect of gal-3 on MMP1 and leptin expression in human OA osteoblasts under different oxygen tensions, to demonstrate signaling pathways which are involved, and finally to identify the ligands on osteoblast surface able to regulate gal-3-induced MMP1 and leptin expression.

RESULTS

Stimulation of MMP1 and leptin expression by gal-3 under hypoxia and normoxia

Gal-3 stimulated MMP-1 expression by 6.2 fold ($p<0.0001$, n=5) under normoxia and by 5.4 fold ($p<0.001$, n=9) under hypoxia versus their respective control (vehicle), (Figures 1A, 1B). As leptin expression is very weak under normoxia[7], its expression was investigated only under hypoxia. Leptin expression was increased by 2.7 fold by Gal-3 ($p<0.0001$, n=9).

Signaling pathways involved in gal-3-induced MMP1 and leptin expression

As shown in figure 2, gal-3 activated the three investigated signaling pathways, namely ERK1/2, P38 MAPK and PI-3K/AKT. The first and highly activated pathway, as soon as 15 min after the addition of gal-3 to osteoblasts, was the PI-3K/AKT one (Figure 2). The second highly activated pathway was ERK1 (p44) / ERK2 (p42) whose phosphorylation peaked after 1 hour of gal-3 challenge. Of note, ERK2 (p42) was already activated to some extent in basal conditions whereas ERK1 (p44) was particularly sensitive to gal-3. Finally, P38 MAPK phosphorylation increased as soon as 15 - 30 min after gal-3 addition but its activation seemed to be weaker than the two

others. Then, we investigated whether these activated signaling routes contributed to the gal-3-induced MMP1 and leptin expression by using well known selective inhibitors (Figure 3). Regarding MMP1 expression under both normoxic and hypoxic conditions, P38 MAPK (SB203580) and PI-3K/AKT (LY294002) were involved whereas ERK1/2 (PD98059) did not regulate MMP1 (Figure 3A and 3C). In addition, MMP1 expression was almost significantly abolished by LY294002 whatever the oxygen concentration was. In contrast, the effect of SB203580 under hypoxic conditions seemed to be less potent than under normoxia. Indeed, gal-3-induced MMP1 expression in the presence of SB203580 was still 2.2 fold under hypoxia compared to 1.3 fold under normoxia. The basal levels of total collagenase 1 measured in osteoblast medium under normoxia or hypoxia were not significantly different indicating that hypoxia did not disturb the capacity of osteoblasts to produce this enzyme (Figure 3B and 3D). In accordance with the results obtained for mRNA levels, gal-3 induced collagenase 1 levels were regulated by P38 MAPK ($p < 0.03$, n=6 and $p < 0.01$, n=8) and PI-3K/AKT ($p < 0.007$, n=6 and $p < 0.02$, n=8) under normoxia and hypoxia, respectively. However in contrast to data obtained at the mRNA level, SB203580 was as powerful on collagenase 1 under hypoxia than under normoxia. Unlike MMP1, leptin expression was completely inhibited by each of the inhibitors both at the mRNA ($p < 0.0001$ for all inhibitors, n=8) and the proteins levels ($p < 0.008$ for PD98059, $p < 0.005$ for SB203580 and $p < 0.004$ for LY294002, n=6, Figure 3E and 3F)

Purification and identification of potential galectin-3 ligands from membrane-enriched fractions of human OA osteoblasts

As illustrated in Figure 4, silver staining on SDS PAGE of the concentrated eluted fraction from the gal-3-Sepharose column, showed numerous bands of variable intensity which an apparent molecular weight ranging from 250 to 10 kDa. The analysis by mass spectrometry identified 38 proteins in total. All these identified proteins had a mascot score above 80 and were therefore considered as putative ligands of gal-3. According to the highest mascot score and because the membrane-enriched fractions could be contaminated by cytoplasm, 24 proteins described in table 2 were retained. Among them, we chose to focus on the β1 and β5 integrins and on the 4F2hc for their previously recognized binding potency to Gal-3 and on CD44 because of its newly identified role as a gal-3 ligand and well known regulatory role in joint cells.

Ligands on osteoblast surface able to regulate Gal-3-induced MMP1 and leptin expression

First of all, the silencing efficacy of siRNAs on each targeted ligand was verified by RT-qPCR. Our data indicated that CD44 was inhibited by 55%, SLC3A2 by 51%, integrin β1 by 80%, and integrin β5 by 70% (data not shown). Under normoxia, gal-3-stimulated MMP1 expression was inhibited by 35% ($p < 0.0001$, $n = 13$) when CD44 was silenced, by 50% ($p < 0.0001$, $n = 13$) with SLC3A2 silencing, and finally by 13% ($p < 0.0001$, $n = 13$) with integrin β1 silencing (Figure 5A). Unlike other ligands, the silencing of integrin β5 stimulated MMP1 expression 2.7-fold in basal ($p < 0.0001$, $n = 6$) and 1.5-fold in gal-3 conditions ($p = 0.0003$, $n = 6$) compared to their respective control. Collagenase 1 quantification under normoxia almost corroborated these results. Indeed gal-3-induced collagenase 1 was down-regulated by 4F2hc and integrin β1 silencing while CD44 silencing was not significantly efficient (Figure 5B). In addition, The ELISA confirmed that integrin β5 silencing upregulated collagenase 1 in basal and gal-3 conditions (Figure 5B). Under hypoxia, MMP1 expression was regulated in a similar fashion (Figure 5C). Leptin expression was significantly inhibited by 80% with SLC3A2 silencing ($p < 0.0001$, $n = 7$), whereas an inhibition of 62% was obtained with integrin β5 silencing ($p = 0.0003$, $n = 7$; Figure 5D). The silencing of either CD44 or integrin β1 induced a stimulation of 1.5 and 1.6 fold, respectively ($p < 0.0001$ for both conditions, $n = 7$)

DISCUSSION

Although intracellular gal-3 was shown to be protective in joint cells[18], its extracellular localization played a deleterious role on joint tissues[5]. During rheumatoid arthritis and OA, the gal-3 concentration increases in synovial fluid[19, 20] allowing its binding to target cells into the knee. In the present study, we demonstrated that gal-3 stimulated differently MMP1 and leptin in OA osteoblasts. The regulation was gene-dependent rather than culture condition-dependent since gal-3-induced cell signaling pathways stimulating MMP1expression in the same manner, both under normoxia and hypoxia. Of note, our data provide evidence that the regulation of MMP1 and leptin was discriminated at the cell surface level since a variable number of ligands activate these two genes. Integrin β1, a well known gal-3 ligand is involved in the regulation of gal-3-stimulated MMP1. Integrin β1 contributes to the differentiating effect of 1,25-dihydroxy $VitD_3$-in osteoblasts[15], and we have previously shown that gal-3

modulates osteocalcin production[5] suggesting that gal-3 could modulate osteocalcin by interacting with integrin β1. In addition, integrin β1 regulates several biological functions of osteoblasts particularly in the early stages of differentiation[21] and at least, when osteoblasts are in culture, via the integrin/PI 3-kinase pathway[22], a pathway also activated by gal-3 in OA osteoblasts. Unlike Integrin-β1, which mediates osteoblast differentiation and osteoblastic ECM formation promoted by cyclic tensile strain, integrin-β5 is not involved in these osteoblast responses[23]. However, we demonstrated here that integrin-β5 is a negative regulator of MMP1 since its silencing induces MMP1 expression independently of gal-3 stimulation. A link between integrin-β5 and MMP1 has previously been demonstrated in primary skin fibroblasts but in a different regulation manner[24]. Indeed, this study demonstrated that CCN3, a ligand of integrin-β5, induced MMP1. Our data raises the hypothesis that a ligand binding on integrin could be no effective on a biological point of view or could potentially inhibit the property of this protein such as a siRNA does. Finally, the last gene studied able to regulate MMP1 is *SLC3A2*, which codes for the protein 4Fh2c. A previous study in Hela cells showed that the binding of gal-3 to 4Fh2c stimulated MMP 2 expression[25]. The 4Fh2c may act alone or in combination with CD147 or integrin β1.

We have previously demonstrated that hypoxia was able to upregulate leptin production in human OA osteoblasts[7]. We clearly showed here that gal-3 enhance the effects of hypoxia on this gene. The regulation leptin by gal-3 implicates the three signaling pathways studied as well as 4Fh2c and integrin β5. Regulation of leptin via gal-3 has never been demonstrated before whereas leptin effects regulated by gal-3 have been described last year. Indeed, leptin-induced collagen production was prevented by inhibiting intracellular gal-3 in cardiac myofibroblasts[26]. We showed also that CD44 and integrin β1 regulate leptin in the presence of gal-3. CD44 is a receptor for hyaluronic acid (HA) but can also interact with collagen, osteopontin and MMPs. CD44 by binding to HA inhibits BMP-induced osteoblastic differentiation[27]. CD44 is also involved in the regulation of subchondral bone osteoblast metabolism by cyclic compression[28]. However to our knowledge, it is the first time demonstrating that CD44 modulates leptin expression in human OA osteoblasts. Therefore, CD44 can be involved in bone remodeling. Whether 4Fh2c could interact with CD44 will need to be investigated in further studies. Integrin β1 was associated with a positive leptin regulation in adipocytes via the ERK pathway[29]. In the present study, we obtained an upregulation of gal-3-induced leptin when integrin β1 was silenced. Therefore, we can

assume that osteoblasts, which are a different cell type than adipocytes, moreover subjected to hypoxia and activated by gal-3 are totally independent conditions than those described by Farnier et al.

As mentioned before, macrophages are able release great amounts of their gal-3 content. These cells are recovered in high numbers in the inflammatory synovium. However, Walsh and colleagues described CD68-positive mononuclear cells in subchondral bone spaces and vascular channels that displayed the appearance of infiltrating macrophages in OA[30]. Interestingly, some CD68-positive multinucleated, osteoclast-like cells were also observed in vascular channels particularly when proliferating endothelial cells were present[30]. Therefore not only synovium but inflammatory flares of vascular vessels within bone could account for the presence of gal-3 in the vicinity of the osteoblasts. This reinforce our hypothesis raised previously[7], that leptin could be involved in the subchondral bone remodeling during OA.

CONCLUSION

We demonstrated that Gal-3 stimulates the MMP 1 production under normoxia and hypoxia, the enzyme involved in bone degradation. Gal-3 induced MMP1 is mainly regulated by binding to 4F2hc and integrin β1. Gal-3 is also able to stimulate leptin production under hypoxia via 4F2hc and integrin β5.

MATERIALS AND METHODS

Human OA specimens

All specimen collection and all procedures were approved by the Ethics Committee of the Nancy University Hospital (CHU) (agreement #UF 9607-CPRC 2005) and conducted in conformity with the declaration of Helsinki. Human osteoblasts from subchondral bone (SCB) of tibia plateaus were obtained from 18 patients (aged 68.4 ± 10.0 years; BMI 31.7 ± 3.3 kg/m^2; 12F/6M) with OA who had undergone total knee arthroplasty. All patients with OA were verified by certified rheumatologists according to the recognized clinical criteria of the American College of Rheumatology[31]. All human material was acquired following a signed agreement by patients who have undergone knee surgery according to the CHU of Nancy Ethical Committee guidelines.

Subchondral bone osteoblast culture

Both cartilage and trabecular subchondral bone (SCB) were removed from tibial plateaus, then preparation of osteoblasts was performed from the SCB plate as described previously[5, 32]. Briefly, the SCB plate was cut into small pieces of 2 mm^2, followed by repeated enzymatic digestions with 1mg/ml de collagenase type I (Sigma aldrich, France) in serum free Dulbecco's modified Eagle's medium (DMEM) at 37°C for 30 min, 30 min and 240 min. After two washings with DPBS, the digested SCB pieces were cultured in DMEM/F12 containing 10% foetal bovine serum (FBS) and a mixture of 1% penicillin-streptomycin. Culture medium was refreshed every two days until confluence. Then, cells were seeded once at 25,000 cells/cm^2 and grown for 5 days before beginning experimentation. Confluent cells were starved for 24h in the same medium containing 0.5% FBS and incubated in the presence of vehicle (PBS with or without 0.1% of dimethylsulfoxide) or rh gal-3 (5µg/ml) in the presence or absence of selective inhibitors, under normoxia (21% O_2) or hypoxia (2% O_2) for 24h. Recombinant human gal-3 was prepared in our laboratory and sterilized on a 0.2 µm filter as mentioned previously[5]. Supernatants were collected at the end of incubation and kept at -20°C prior to assays.

RT-qPCR assays

Total RNA was extracted with QIAzol lysis reagent (Qiagen, France) according to the manufacturer's protocol. Two hundred ng of total RNAs were reverse-transcribed for 90 minutes at 37°C in a 20 µl reaction mixture containing 10 mM dNTP, 5 µM random hexamer primers, 1.5 mM $MgCl_2$, and 100 U Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, France). cDNAs production was performed in a Mastercycler gradient thermocycler (Eppendorf, France). Next, real time PCR was performed by the Step One Plus technology (Applied Biosystems, France) using specific primers (Table 1) and iTAQ SYBRgreen master mix system (Biorad, France). All reagents used for RT-PCR were added at the concentrations recommended by the manufacturer. Melting curve was performed to determine the melting temperature of the specific PCR products. The mRNA levels of the gene of interest and of the *ribosomal protein 29* (*RPS29*), chosen as housekeeping gene, were determined in parallel for each sample. Quantification was determined using the ΔΔCt method and the results were expressed as fold expression over the appropriate control.

Western immunoblotting

Cells were lysed in 1X Laemmli buffer and proteins loaded on 4-20% SDS-polyacrylamide precast gels (BioRad). Immunoblotting as well as signal detection were performed as previously described[33]. Briefly, after 2h in blocking buffer (Tris 50 mM buffered 150 mM saline pH 7.4 (TBS) containing 0.1% of Tween (T-TBS) supplemented with 5% of non-fat dry milk), membranes were washed three times with T-TBS and incubated overnight at 4°C with rabbit primary antibodies. We used antibodies against phosphorylated and total forms of the following MAPK (p-Akt, Akt, p-p38 MAPK, p38 MAPK, p-ERK, ERK; all diluted at 1:1000, Cell Signaling Technology, USA) and β-actin (1:8000, Sigma-Aldrich). After three washings with T-TBS, each blot was incubated for 1h at room temperature with anti-rabbit IgG conjugated with HRP (Cell Signalling Technology) at 1/10000 dilution in blocking buffer. After four additional washings, protein bands were detected by chemiluminescence according to manufacturer's recommendations (Cell Signalling Technology).

Signaling pathway analyses

To evaluate the signaling pathway supporting gene expression induced by gal-3, OA osteoblasts were pre-incubated with either PD98059 (inhibitor of mitogen-activated protein kinase kinase-1 (MEK-1); final concentration 5μM), SB203580 (inhibitor of p38 mitogen-activated protein kinase; final concentration 2μM) or LY294002 (inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase); final concentration 10μM), or the vehicle (DMSO, 1 μl/1000 μl) for 2 h and then incubated for 22 h in presence or absence of rh gal-3. Conditioned media were collected at the end of incubation for the measurement of MMP 1 and leptin levels (see below). In another experiment, cells were pre-incubated with the above inhibitors of cell signalization for 1h and then, stimulated or not with rh gal-3 for 8 hours.

Evaluation of MMP 1 and leptin production

Human total MMP1 and leptin levels were evaluated in conditioned media of osteoblasts using commercially available ELISA kits (DuoSet, R&D Systems, France). The range of measure was 156 to 10000 pg/ml for MMP1 and 31.3 to 2000 pg/ml for leptin.

Screening of potentials ligands in Gal-3 sepharose

Osteoblasts (7.5×10^7 cells) were homogenized in 500 µl of cold 5 mM Hepes pH 7.4 buffer containing 0.25 M saccharose, then sonicated at 4000 Hz (BANDELIN sonoplus GM70) on ice for 3×10 seconds with vortexing for 20 seconds between sonicating periods. Heavy cell debris were removed by centrifugation at 1000 g for 10 min and then, the supernatant was centrifuged at 100000 g for 1 hour. The pellet, which contained the membrane-enriched fractions, was solubilized in Hepes saccharose buffer containing 0.05% TritonX100 and the solubilized fraction was cleared with a centrifugation at 10000 g for 10 min. Purified rhGal-3 (50 mg) was coupled to 10 ml of NHS-activated Sepharose 4 Fast Flow (GE Healthcare Life Sciences, France) according to the manufacturer's direction. The solubilized fraction was applied to the gal-3-Sepharose column, then, the column was rinsed with a Tris saline buffer (20 mM Tris, 0.5 M NaCl pH 7.5) before elution of gal-3 ligands with the same buffer containing 150 mM lactose (Sigma, France).

Mass spectrometry analysis

Eluted proteins were precipitated with Trichloroacetic acid, rinsed twice with acetone and then suspended either in 100 µl Urea buffer (6M urea, 50 mM Tris, pH 7.0) for total digestion and downstream mass spectrometry analysis or in 20 µl SDS PAGE loading buffer for electrophoresis and in-gel digestion. For direct digestion, 10 µl aliquots were used. Cysteines were reduced with 0.4 µl DTT, 100 mM for 45 min at room temperature, alkylated with 0.4 µl iodoacetamide (IAA), 1M, for 45 minutes and the remaining IAA was reduced with 0.4 µl DTT, 1M. Samples were then diluted to 100 µl with Tris, 50 mM, pH 7.0 and 100 ng Trypsin (Promega, sequencing grade) was used for overnight digestion at 37°C. For in-gel digestion, gel bands of interest were excised and processed as follows for protein content identification: cysteines were reduced in DTT, 30 mM, prepared in 100 mM ammonium bicarbonate (BA), and alkylated in IAA 30 mM in BA, each for 45 min at RT. After 2 washings in BA and BA/acetonitrile, 1:1, 15 min each, gel bands were dried in a speed-vac for 1 hour, and digested with 100ng trypsin in 20 µl BA overnight at RT. Peptides were extracted twice consecutively in 25 µl acetonitrile, 80%, TFA 1% for 10min under sonication in a water bath sonicator. Extracted peptides were pooled, dried in a speed-vac and resuspended in 8 µl 2% acetonitrile, 0.1% TFA. HPLC was performed using an Ultimate 3000 equipment (Dionex). Peptides (6.4 µl) were injected in the microliterpickup mode onto a desalting

column and loaded onto a 15 cm Acclaim pepmap RSLC C18 column (dionex) and eluted over a 60min run by a 2-45% acetonitrile linear gradient. A total of 170 fractions (for in-gel digests) or 340 fractions per sample was collected onto a 1584 MTP anchorchip MALDI plate via a Proteineer FcII fractionator (Bruker) Fractions were mixed with α-Cyano-4-hydroxycinnamic acid (HCCA) directly upon deposition. Sample acquisition in TOF and TOF/TOF modes was performed automatically using WARP-LC as pilot software on an Autoflex speed MALDI mass spectrometer (both from Bruker). Peptide assignments, protein identification and scoring were managed on a Proteinscape server allowing a 50 ppm tolerance for mass measurements through interrogation of the whole non-redundant NCBInr database of a local Mascot server. Proteins with mascot scores above 80 were considered significant.

Knockdown by siRNA

siRNAs (flexitube siRNA) targeting human *CD44, SLC3A2* and *Integrin ß5* gene were purchased from Qiagen, (France) whereas those targeting *Integrin ß1* gene and the non-silencing control siRNA (negative Control, medium GC content) were obtained from Eurogentec (France). Transfections were carried out with each siRNA at a final concentration of 20 nM, using INTERFERin^{TM} (Polyplus-transfection SA, France). siRNAs were diluted in serum-free medium prior to addition of INTERFERin^{TM} for 10 min at room temperature. OA osteoblasts seeded in 6-well plates at 25,000 cells/cm^2 were washed with PBS, replenished with fresh medium and then, the siRNA-INTERFERin^{TM} mix was added to cells for 48 h. After a 6 h of starvation in DMEM/F12 medium containing 0.5% of FBS, cells were incubated in the presence or absence of rh gal-3 (5µg/ml) for another 24h. Knockdown efficiencies were evaluated by RT real time PCR.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using either one way ANOVA followed with Dunnett's multiple comparison *post-hoc* test or t-test using GraphPad Prism 5 (GraphPad Software). P values <0.05 were considered significant among different experimental groups.

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Legends of figures

Figure 1: MMP 1 and leptin expression under normoxia and hypoxia. Human OA osteoblasts were incubated for 24 hours under either normoxia (21% O_2) or hypoxia (2% O_2) in the presence of 5 µg/ml galectin-3 (Gal-3) or vehicle (PBS). Gene expression was assessed by RT-qPCR. *= P <0.05.

Figure 2: Screening of signaling pathways induced by Gal-3 under normoxia. Cells were incubated with 5 µg/ml of Gal-3 for 0 to 24h. (A) The phosphorylation of AKT, P38 MAPK and ERK1/2 was assessed by immunoblotting. β-actin served as control of loading.

Figure 3: Signaling pathways involved in Gal-3-stimulated MMP 1 and leptin expression under normoxia and hypoxia. Cells were pretreated with vehicle (0.1% dimethyl sulphoxide) or with different inhibitors for 2 hours under normoxia, then Gal-3 was added into wells for the remaining 22 hours under normoxia and hypoxia (n = 8 experiments). MMP1 expression was assessed by RT-qPCR (A, C) and ELISA (B, D) whereas leptin expression was measured by RT-qPCR only (E). PD: PD98059, mitogen-activated protein kinase kinase (MEK) inhibitor (5µM); SB: SB203580, p38 mitogen-activated kinase inhibitor (2µM); LY: LY294002, Phosphoinositide 3-kinase inhibitor (10µM). * = P <0.05 vs vehicle alone (DMSO). # = P <0.05 vs vehicle (DMSO) + Gal-3.

Figure 4: Purification of cell surface ligands recognized by galectin-3. The protein fraction eluted by lactose from the Gal-3 sepharose column was concentrated, migrated on SDS-PAGE and stained with silver nitrate (representative gel of 2 independent experiments).

Figure 5: Ligands on osteoblast surface able to regulate Gal-3-induced MMP1 and leptin expression. Cells were treated with 20 nM of SiRNA targeting the gene of interest for 48h, then Gal-3 was added or not for another 24h under normoxia (n = 13) or hypoxia (n = 7). MMP1 (A) and leptin (B) expression was measured in by RT-qPCR. siCtrl: Scrambled siRNA, siITGB1: integrin β1, siITGB5: integrin β5. * = P <0.05 gal-3 stimulation vs the corresponding control. # = P <0.05 vs siCtrl + Gal-3. $ = P <0.05 vs siCtrl alone.

Table 1. Oligonucleotides used for real time RT-PCR

Genes		Sequences 5'-3'	Gene ID
RPS29	S	GGG-TCA-CCA-GCA-GCT-CGA-GA	NM_001032.3
	AS	CAG-ACA-CGA-CAA-GAG-CGA-GA	
MMP1	S	AGG-TCT-CTG-AGG-GTC-AAG-CA	NM_002421.3
	AS	CTG-GTT-GAA-AAG-CAT-GAG-CA'	
LEPTIN	S	GGC-TTT-GGC-CCT-ATC-TTT-TC	NM_000230.2
	AS	GGA-TAA-GGT-CAG-GAT-GGG-GT	
SLC3A2	S	GAC-CCC-TGT-TTT-CAG-CTA-CG	NM_001012662.2
	AS	TCA-GGG-AAG-CTG-GAC-TCA-TC	
CD44	S	GAC-AAG-TTT-TGG-TGG-CAC-G	NM_000610.3
	AS	CAC-GTC-GAA-TAC-ACC-TGC-AA	
Integrin β1	S	TTT-GAG-CAA-ACA-CAC-AGC-AA	NM_002211.3
	AS	GAG-TCG-CGG-AAC-AGC-AG	
Integrin β5	S	CCT-TTC-TGT-GAG-TGC-GAC-AA	NM_002213.4
	AS	CCT-TTC-TGT-GAG-TGC-GAC-AA	

Table 2

Protein	MW	Score	Gene
Aminopeptidase	109.5	1409.2	ANPEP
Integrin beta-1	88.4	669.6	ITGB1
5'-nucleotidase	63.3	518.8	NT5E
CD44	81.5	479.5	CD44
HLA class I histocompatibility antigen, A-3 alpha chain	40.8	460.3	HLA-A
HLA class I histocompatibility antigen, A-69 alpha chain	41	446.3	HLA-A
HLA class I histocompatibility antigen, B-55 alpha chain	40.5	395.1	HLA-B
C-type mannose receptor 2	166.6	390.9	MRC2
4F2 cell-surface antigen heavy chain	68	390.6	SLC3A2
Integrin beta-5	88	356.7	ITGB5
CD166	65.1	315.6	ALCM
Sodium/potassium-transporting ATPase subunit alpha-1	112.6	205.5	ATP1A1
Neuroplastin	44.4	180	NPTN
Basigin	22.1	162.9	BSG

Integrin alpha-V	116	151.3	ITGAV
Myoferlin	234.6	146.9	FER13
Prolow-density lipoprotein receptor-related protein 1	504.3	144.9	LRP1
Ectonucleotide pyrophosphatase/phosphodiesterase family member 1	104.9	141.7	ENPP1
Cytoskeleton-associated protein 4	66	112.6	CKAP4
Trophoblast glycoprotein	46	102.8	TPBG
Integrin alpha-11	133.4	97.8	ITGA11
CD276	57.2	92.3	CD276
Gamma-glutamyltransferase 5	62.2	86.2	GGT5
Thy-1 membrane glycoprotein	17.9	82.3	THY1

Figure 1

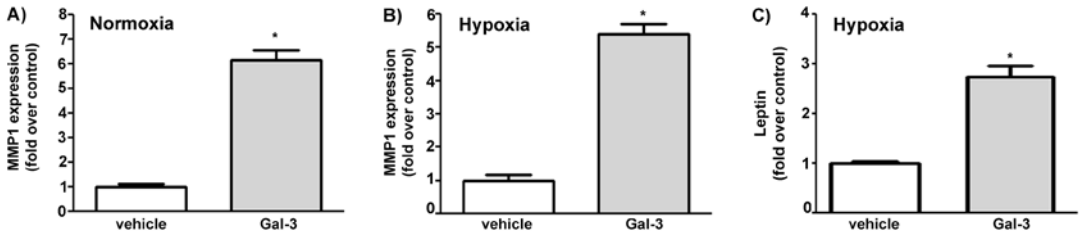


Figure 2

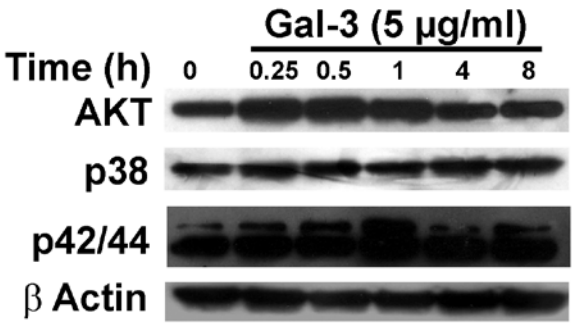


Figure 3

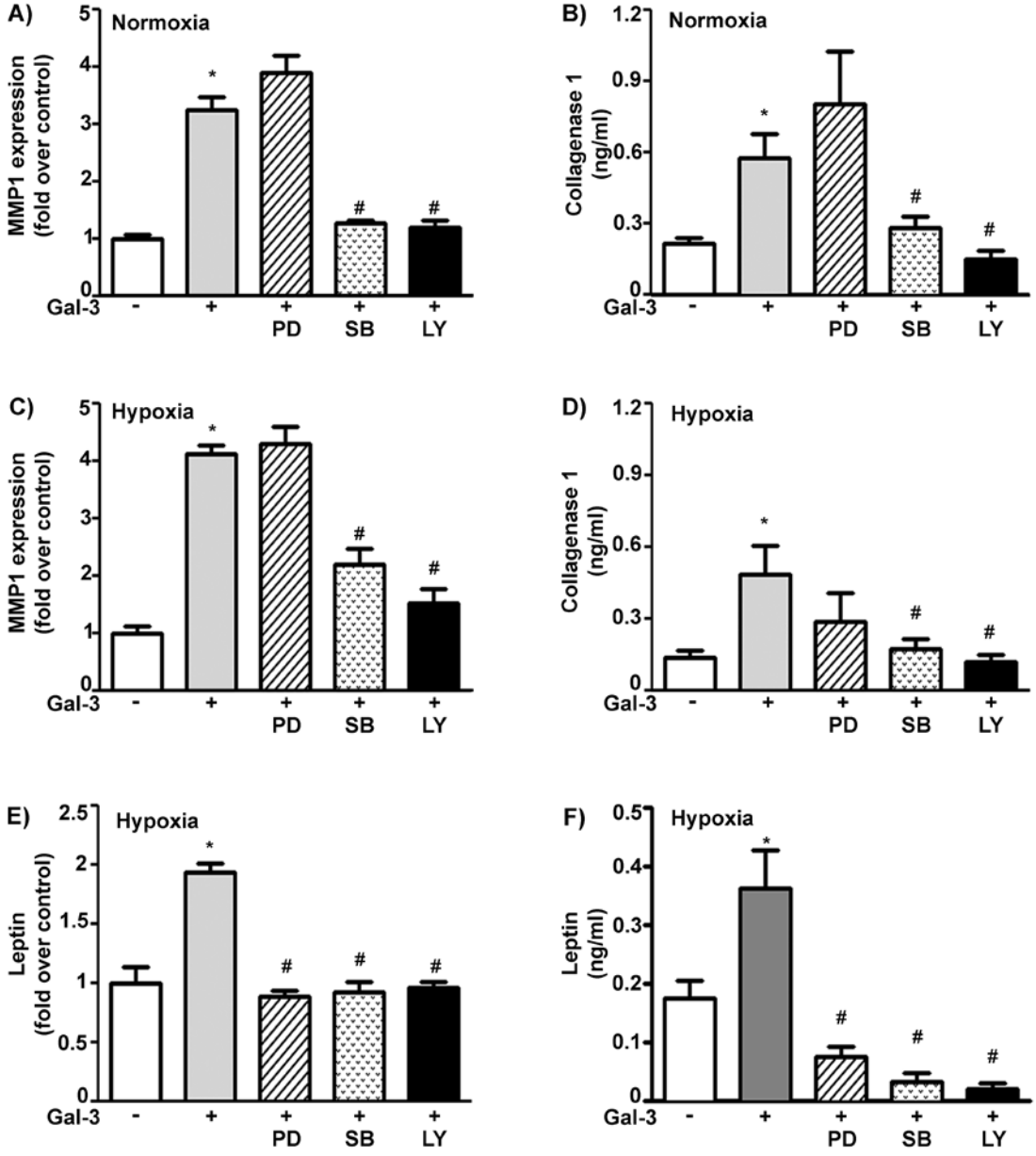


Figure 4

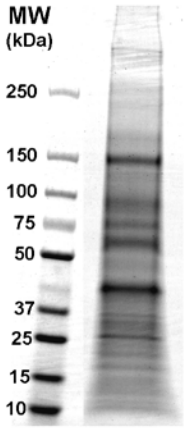
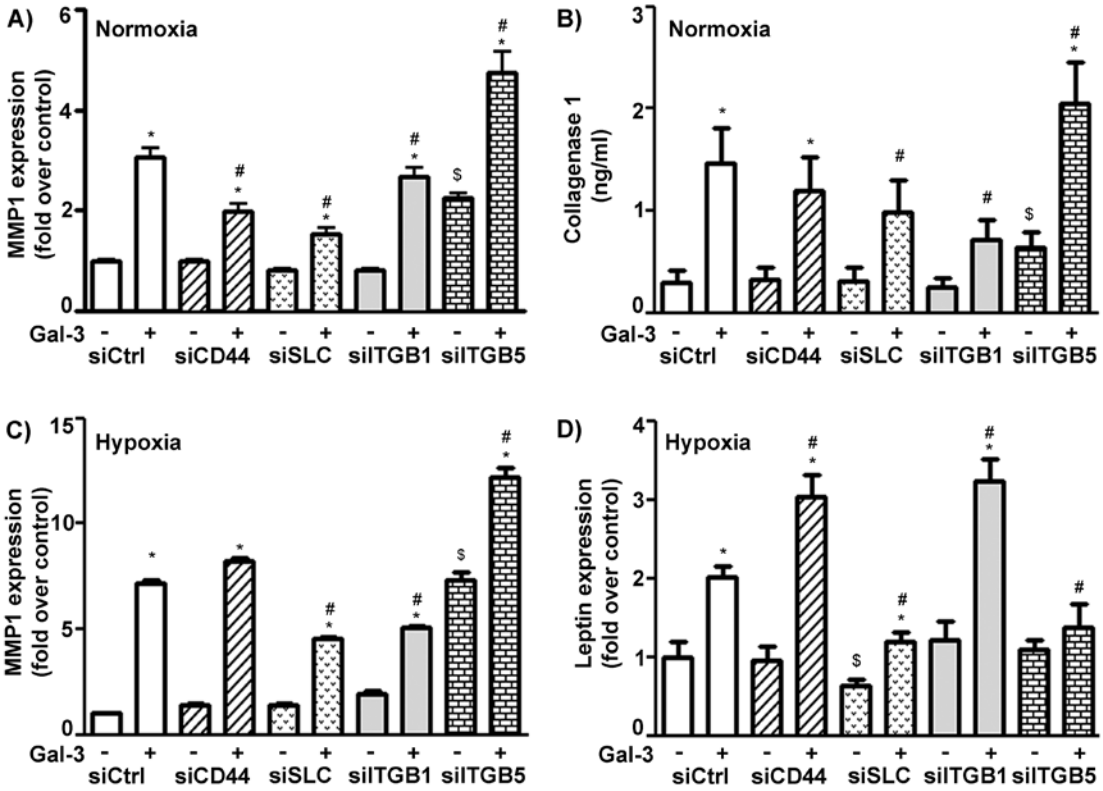


Figure 5



Materials and methods (supplement data 2)

3.4 Other genes expression modified by Gal-3

We also studied the expression of CYP27B1 and Gamma-glutamyl carboxylase (GGCX) under normoxia and hypoxia, in presence of inhibitors or not (Figure 3.5, 3.6). Cells preparation, treatment and gene expression are carried out as described in the article for MMP-1 and leptin.

3.5 Mineralization

3.5.1 Cell culture conditions

First passage human OA osteoblasts were seeded at 25000 cell/cm^2 in 24 well plates and cultured in DMEM F12 containing 10% SVF and 1% penicillin/streptomycin (complete medium). When cells reached confluence (day 0), mineralizing experiments were started by incubating cells under normoxia (21% O_2) or hypoxia (2% O_2) in the 7 different following conditions:

PBS Gal-3 (1.5 µg/ml or 5 µg/ml) $VitD_3$ (50 nM)
$VitD_3$ (50 nM) + Gal-3 (5 µg/ml) Dexamethasone (DEX, 10^{-8} M)
DEX (10^{-8} M) + Gal-3 (5 µg/ml) $VitD_3$ + DEX

For mineralizing experiments, the complete DMEM F12 contained β-glycerophosphate (10 mM) and ascorbic acid (50 µg/ml) was changed every 2 days for a total of 28 days.

The same experiments were applied to Saos-2 for 21 days. Saos-2 is a cell line derived from the primary osteosarcoma which possess several osteoblastic features. These cells served as a positive control for their capacity of mineralization.

3.5.2 Alizarin red staining (Ca^{2+} staining)

At the end of the experiments described above, cells were rinsed twice with and then fixed in 96% alcohol for 30 minutes. After 2 other washes in 0.9% NaCl, wells were filled with 0.5 ml of a 2% of alizarin red (diluted in H_2O, pH 4.1-4.3) for 5 minutes.

Plates were washed twice with water, inverted onto paper towels and left dry at room temperature (Figure 3.7).

3.5.3 Dye extraction and quantity evaluation of mineralization

Eight hundred µl of 10% (v/v) acetic acid were added to each well, then, the plate was incubated at room temperature for 30 minutes under shaking. The monolayer, which is now loosely attached to the plate, was scraped with a tip of 1000 µl and transferred with the acetic acid solution into a 1.5 ml micro-centrifuge tube. After having used a vortex for 30 seconds, the slurry was heated to 85°C for 10 minutes and cooled down in ice for 5 minutes. The slurry is then centrifuged at 13000 g at 4°C for 15 minutes and 500 µl of the supernatant was transferred to a new 1.5 ml micro-centrifuge tube. Thereafter, 100 µl of freshly prepared solution of 10% (v/v) ammonium hydroxide was added to neutralize the acid. Aliquots (150 µl) of the supernatant were read in triplicates at 425 nm in a 96-well plate (Figure 3.8).

Table 3. 1 Oligonucleotides used for real-time RT-PCR

Gene		Sequence 5'-3'	Gene ID
RP29	S	5'-GGG-TCA-CCA-GCA-GCT-GTA-CT-3'	NM_001032.3
	AS	5'-CCG-ATA-TCC-TTC-GCG-TAC-TG-3'	
MMP 1	S	5'-AGG-TCT-CTG-AGG-GTC-AAG-CA-3'	NM_001145938.1
	AS	5'-CTG-GTT-GAA-AAG-CAT-GAG-CA-3'	
Leptin	S	5'-GGC-TTT-GGC-CCT-ATC-TTT-TC-3'	NM_000230.2
	AS	5'-GGA-TAA-GGT-CAG-GAT-GGG-GT-3'	
SLC3A2	S	5'-GAC-CCC-TGT-TTT-CAG-CTA-CG-3'	NM_001012662.2
	AS	5'-TCA-GGG-AAG-CTG-GAC-TCA-TC-3'	
CD44	S	5'-GAC-AAG-TTT-TGG-TGG-CAC-G-3'	NM_000610.3
	AS	5'-CAC-GTC-GAA-TAC-ACC-TGC-AA-3'	
Integrin β1	S	5'-TTT-GAG-CAA-ACA-CAC-AGC-AA-3'	NM_002211.3
	AS	5'-GAG-TCG-CGG-AAC-AGC-AG-3'	
Integrin β5	S	5'-CCT-TTC-TGT-GAG-TGC-GAC-AA-3'	NM_002213.4
	AS	5'-TGT-AAC-CTG-CAT-GGC-ACT-TG-3'	
CYP27B1	S	5'-CCT-GGC-AGA-GCT-TGA-ATT-GCA-3'	NM_000785.3
	AS	5'-GGG-GAA-GAT-GTA-TAC-CTT-GGT-3'	
GGCX	S	5'-CAT-TCC-TCC-TAT-GGG-CAT-TC-3'	NM_000821.5
	AS	5'-CTG-TAT-GGG-TTG-TTG-GCC-TT-3'	

Results (supplement data 2)

3.4 Other genes expression modified by Gal-3

3.4.1 Modification of CYP27B1 and GGCX expression by Gal-3

Under normoxia, in the presence of Gal-3, the expression of CYP27B1 and GGCX is stimulated by 3.9 fold (Figure 3.5 A, n = 4, $p<0.05$) and 4.4 fold (Figure 3.5 C, n = 5, $p<0.05$) respectively. Under hypoxia, Gal-3 inhibits the expression of CYP27B1 (Figure 3.5 B, n = 4, $p<0.05$), while still stimulates the expression of GGCX by 2.8 fold (Figure 3.5 D, n = 6, $p<0.05$).

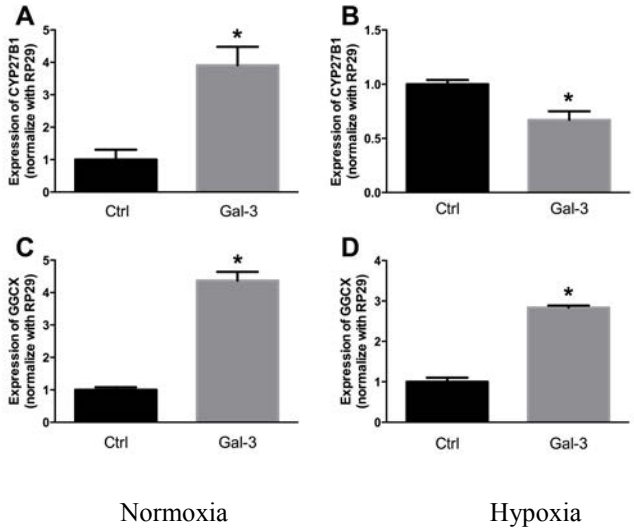


Figure 3. 5: The modification of gene expression by Gal-3 under normoxia and hypoxia. 1st passage of osteoblast are treated with Gal-3 or not for 24 h, CYP27B1 expression under (A) normoxia (B) hypoxia and GGCX expression under (C) normoxia and (D) hypoxia are evaluated by qPCR. *P<0.05.

3.4.2 Signaling pathways involved in the modification of CYP27B1 and GGCX expression by Gal-3

On the whole, ERK1/2, p38 MAPK and PI3-K/AKT all are involved in the modification of CYP27B1 and GGCX expression by Gal-3. For CYP27B1 expression, PD, SB and LY reverse the effect of Gal-3 by 50% (Figure 3.6 A B, n = 5, $p<0.05$). For GGCX expression under normoxia, SB and LY inhibit the stimulation by Gal-3 by 20% and 50% respectively, while PD presents little inhibition regardless of significance

(Figure 3.6 C, n = 5, $p<0.05$). Under hypoxia, in the presence of Gla-3, PD inhibits the GGCX stimulation by 20%, while by 40% for SB and LY (Figure 3.6 D, n = 4, $p<0.05$). DMSO strongly influence the stimulation of GGCX by Gal-3 whereas almost show no effects on the CYP27B1 expression.

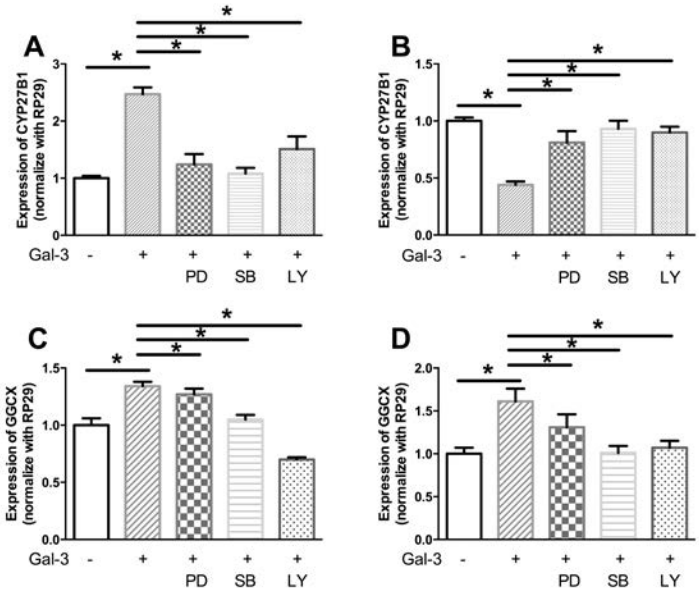


Figure 3. 6: Signaling pathways involved in modification of CYP27B1 and GGCX expression by Gal-3 under normoxia and hypoxia. (A, B) Cells were pretreated with vehicle (0.1% DMSO) or different inhibitors for 2 hours, then Gal-3 (5 μg/ml) was added into cells for the rest of 22 hours, CYP27B1 expression (n = 5) under normoxia (A) and hypoxia (B), GGCX expression under normoxia (n = 5) (C) and hypoxia (n = 4) were measured by qPCR. PD: PD98059, inhibitor of mitogen-activated protein kinase kinase (MEK); SB: SB203580, p38 mitogen-activated kinase inhibitor; LY: LY294002, Phosphoinositide 3-kinase inhibitor. *P <0.05.

3.5 Mineralization

3.5.1 The mineralization of OA osteoblast and Saos-2

OA osteoblasts and Saos-2 are colored by Alizarin red to evaluate the mineralization on the 28th day and 21st day respectively. On the whole, more mineralization has been found in either OA osteoblast or Saos-2 under normoxia compared with that under hypoxia. Under either oxygen tension, $VitD_3$ inhibits the mineralization compared with Ctrl.

For osteoblasts, when Gal-3 was added, the inhibition of VitD3 on mineralization was ameliorated, particularly under hypoxia (Figure 3.7 B). On the other hand, Gal-3 (1.5 μg/ml) induces more mineralization than $VitD_3$ + Gal-3 or $VitD_3$. However, it is difficult to conclude that obvious differences of mineralization exist in various conditions.

In contrast to osteoblasts, Saos-2 are more be able to mineralize, and differences on mineralization in each condition are more obvious under hypoxia (Figure 3.7 D). Compared with Ctrl, Gal-3 promotes the mineralization, and this promotion seems to be more clear in high concentration of Gal-3 (5 μg/ml). DEX increased the mineralization than Ctrl, which was aggravated by the addition of Gal-3.

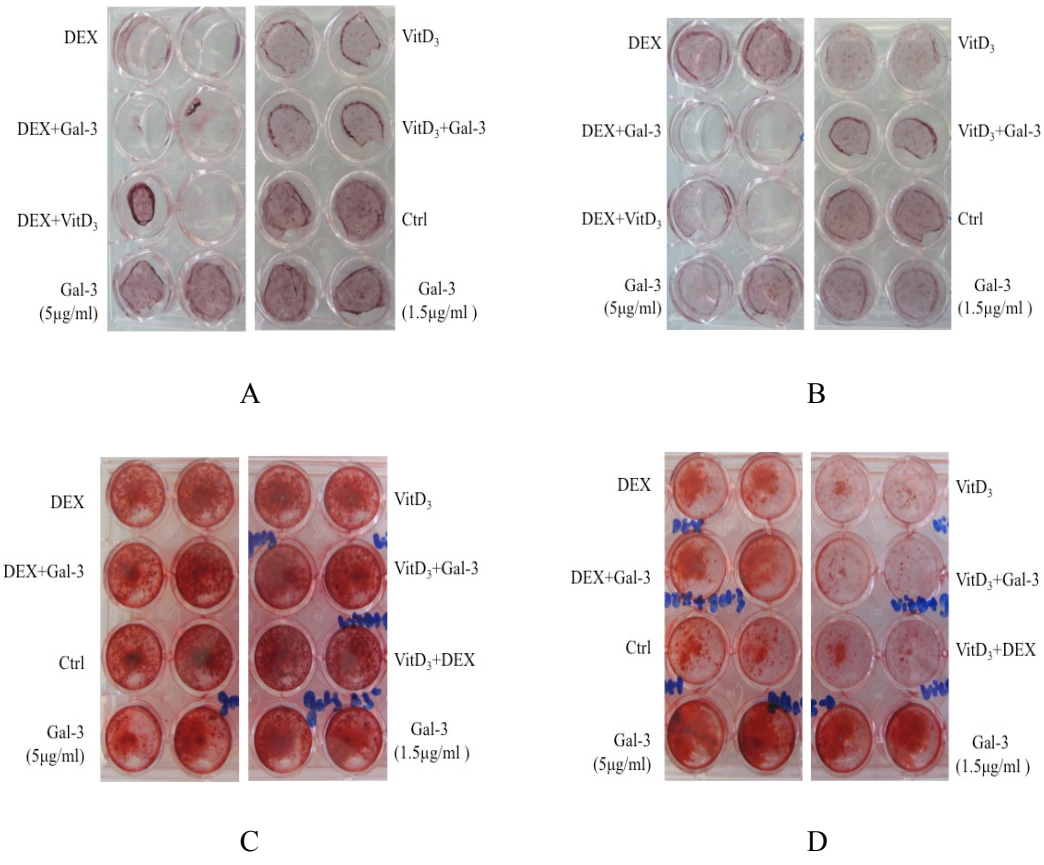


Figure 3. 7: Mineralization staining with OA osteoblasts (A, B) and Saos-2 (C, D) by Alizarin Red. Osteoblasts and Saos-2 received treatments for 28 or 21 days, respectively, under normoxia (A, C) or hypoxia (B,D).

3.5.2 Quantification of osteoblast and Saos-2 mineralization

The dye absorbance in osteoblasts and Saos-2 reflects their mineralization differences, with absorbance below 0.15 and around 2 respectively, which corresponds with the coloration morphology.

For osteoblasts, under normoxia (Figure 3.8 A), we conclude again that $VitD_3$ reduced the mineralization compared with Ctrl, Gal-3 promotes the mineralization compared with $VitD_3$ + Gal-3. These alterations confirmed the inhibition of $VitD_3$ on mineralization. Under hypoxia (Figure 3.8 B), it is difficult to conclude the differences in various conditions.

For Saos-2, we can not make conclusions under normoixa (Figure 3.8 C). It seems that compared with Ctrl under hypoxia (Figure 3.8 D), there is a trend of mineralization augmentation only in presence of Gal-3, particularly at its high concentration (5 µg/ml).

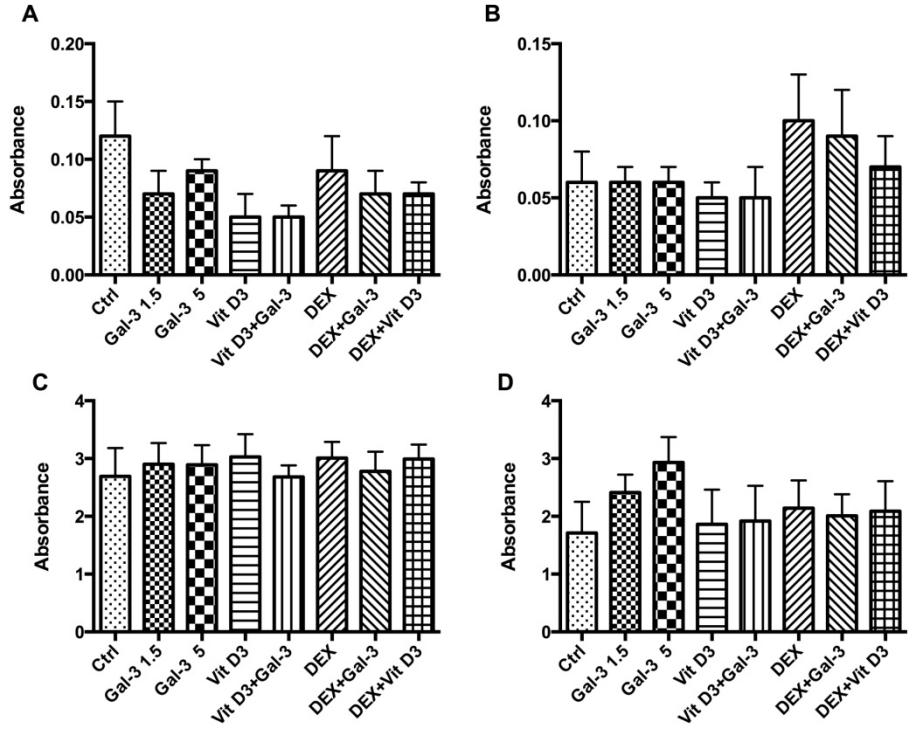


Figure 3. 8: Quantification of osteoblast and Saos-2 mineralization. Osteoblasts (n = 3) and Saos-2 (n = 4) received treatments for 28 or 21 days, respectively, under normoxia (A, C) or hypoxia (B,D).

CHAPTER IV

GENERAL DISCUSSION

GENERAL DISCUSSION

OA, characterized by pain and loss of joint function, is the most common joint disease worldwide. In the advanced stage of OA, the paradox between bone sclerosis (osteogenesis) and hypomineralization still makes us confused. An abnormal behavior and metabolism of OA osteoblasts has been demonstrated, represented by several different genes expression related to osteoblast function, bone remodeling and mineralization. Many of these genes are components of TGF-β/BMP and Wnt signaling pathway[425]. Actually, in experimental murine OA models, elevated TGF-β expression and activity was observed to induce osteophytes similar to those found in OA. These observations were further confirmed by clinical reports[251,288]. In contrast, the canonical Wnt/β-catenin signaling pathway, a crucial pathway for osteogenesis, is reduced in OA osteoblasts as compared to normal osteoblasts. This reduced signaling could be linked to abnormal level of Wnt related molecules, such as Rspo-2, Wnt-3a, Wnt-5b and DKK2[286,426].

Osteocalcin (OCN) is a marker of the mature osteoblast, represented by its regulation of mineralization and involvement in the promotion of osteoblast differentiation and activation[427-430]. In our study, we have discovered two populations of osteoarthritic osteoblasts. One population is present with high basic level of OCN, while the other exhibits low basic OCN level but similar capacity to the former in responding to $VitD_3$. Since more differentiated osteoblasts produce higher OCN level in osteoblasts, this OCN level is considered to represent the differentiation condition of OA osteoblasts. Therefore, we could propose that osteoblasts from different OA patients are not in the same differentiated condition. However this hypothesis needs to be verified in large cohort studies.

In addition, it has been reported that the difference of OCN induction in osteoarthritic osteoblast in responding to $VitD_3$ is proportional to the degree of joint damage[431,432]. In our study, though osteoblasts are present with various basic OCN level, the final amount of OCN expression in responding to $VitD_3$ is the same. Therefore, the difference in differentiated condition of osteoblasts could be overcome by $VitD_3$, indicating the abnormal osteoarthritic osteoblast behavior includes altered responses to systemic or local factors[431].

In fact, a previous research has distinguished the osteoarthritic osteoblasts. Based on

responses to PGE_2, two populations of osteoblasts, "High-PGE_2 responsive OA" and "low-PGE_2 responsive OA", have been proposed by Massicotte *et al*[423]. In "High-PGE_2 responsive OA" patients, elevated IL-6 expression and reduced Rspo-2 expression has been found[423,426]. Both PGE_2 and IL-6 are involved in bone resorption, while Rspo-2 is an agonist of Wnt. Therefore, this might explain that High-PGE_2 responsive OA osteoblasts promote bone resorption, while low low-PGE_2 responsive OA osteoblasts favor bone formation.

As high responders to $VitD_3$ have initially low OCN expression level, this lower advanced differentiated state of osteoblasts could be explained by their potential to express Wnt5b levels and other agonists of Wnt pathway. This hypothesis is plausible even though DKK2 (an antagonist of Wnt pathway) expression is increased in high responders. Indeed, the Wnt5b/DKK2 ratio is 2.5 times than that of low responders, which means the differentiation of osteoblasts is inhibited finally. Furthermore, the less differentiated osteoblasts express more TGF-β1, which corresponds to the possibility of inhibition of the late maturation of osteoblasts as proposed previously[433]. Therefore, the identification of different populations of osteoarthritic osteoblasts is crucial in the contribution of clarifying the different responses in OA patients, which suggests the importance of considering individual differences in treating the disease.

Bone sclerosis in OA is attributed to the increase of material density instead of mineral density. This abnormal mineralization was proposed to be correlated with OCN. In addition, Gal-3 was demonstrated to inhibit the OCN expression in osteoblasts. Therefore, we suspected that Gal-3 may contribute to the hypomineralization during OA. Unfortunately, regardless of the regulation on OCN expression in our study, we observed that Gal-3 is not able to modify the mineralization of osteoarthritic osteoblasts *in vitro* which could be attributed to their intrinsic deficiency in mineralization. However, Saos cells whose capacity to mineralize is widely recognized, did not respond to Gal-3 in a mineralization point of view. The possible interpretation of these data is that during the long study period of mineralization, the capacity of regulation on OCN expression by Gal-3 is attenuated or disappeared.

In our study, we also demonstrated that Gal-3 is able to stimulate the leptin expression in hypoxia. This is an adipokine mainly produced by white adipose tissue in controlling

the food intake and energy expenditure, but its role in the regulation of skeletal biology is more appreciated now[434]. As sharing common structural properties with IL-6, leptin is also classified as an adipocytokine which hints its inflammatory potential[435]. In OA, elevated leptin expression has been found in cartilage, synovial fluid and osteophytes[64,65,290]. Low oxygen tension (2% O_2) induced leptin production in human osteoblasts[424]. A correlation of leptin to disease severity in OA patients and animal models has been proposed. The role of leptin in the OA progression is now widely accepted at present. Therefore, during the inflammatory phase of OA, the osteoblasts' differentiation could be inhibited by Gal-3 through regulating the expression of leptin.

Compared to the impact of inflammation in OA, the vascular pathology is less emphasized. Either by vascular occlusion, micro-emboli production or blood stasis, a condition of low-oxygen tension called hypoxia has been proposed. This condition could be enhanced by bone marrow edema-like lesions in increasing the intraosseous pressure. Some areas of joint tissue such synovial fluid and/or subchondral bone possibly suffer from hypoxia, the combination of hypoxia and tissue inflammation may trigger more secretion of gal-3. As previous results showed that basal expression of leptin in osteoblasts under normoxia was almost negligible[424], we did not conduct the experiments on leptin under normoxia.

In contrast to the regulation on OCN expression which is solely controlled by the PI3K pathway, the leptin stimulation by gal-3 is also regulated by ERK1/2 and p38 MAPK. This suggests that even in the same cell type, the effects of gal-3 are exerted in various mechanisms. Among the ligands investigated, 4Fh2c seems mainly responsible for the stimulation of leptin expression by gal-3. However, integrin β5 is also partly involved. 4F2hc has been considered as the ligand of Gal-3 in many cells, such as macrophages[436,437], human jurkat T-cell[438] and myeloid derived suppressor cells[439]. A recent report shows that Gal-3 stimulates the expression of MMP-2 through PI3K/AKT pathway in Hela cells, the association of gal-3 with 4F2hc and the involvement of integrin was proved to play a prominent role[440]. In addition, gal-3 mediates the endocytosis of β1 integrin in breast carcinoma cells in a lactose-dependent manner, and the binding of gal-3 with integrin β1 induced T-cell apoptosis[394,441]. Though integrin β1 was considered to play a major and complex role in osteoblastic differentiation[442], no report informs us that integrin β1 served as gal-3's receptor on osteoblasts' surface and therefore transduced gal-3's effects in OA. Correspondingly, among the 20 ligands

identified with the gal-3 Sepharose column, β1 and β5 subunits of β integrins were discovered and targeted by siRNA. Therefore, we proposed that 4Fh2c and integrin β5 act predominantly, or under the help of other proteins not identified. Not only β1 and β5, but αv and α1 subunits of integrins which generally heterodimerize with β subunits are also identified in the 20 ligands eluted from gal-3 Sepharose column indicating that they can also be targeted by siRNA to understand their roles in the gal-3's effects.

In addition to modifying the phenotype of osteoblasts, we also demonstrated that gal-3 stimulates the production of collagenase 1 (MMP1), an interstitial collagenase which is responsible for the type 1 collagen degradation[443]. Kaspiris *et al*. report recently that MMP-1 expression was stimulated in advanced OA stage, due to its association with the development of subchondral cysts in specimens from OA patients[444]. It seems that the regulation of MMP1 by gal-3 is cell specific, since this regulation was not demonstrated in human chondrocytes, but MMP3 is stimulated by gal-3 in these cells. Until now, the effects of extracellular gal-3 on MMP1 haven't been demonstrated. However, it has been reported that MMP1 can be regulated by intracellular gal-3 through the association with AP1 complex[445,446]. Therefore, we question whether the extracellular gal-3 is internalized to stimulate the expression of MMP1. However results obtained with the research on ligands seem to deny the hypothesis. Regardless oxygen tension, only p38 MAPK and PI3K are involved in the stimulation of MMP1 expression by gal-3. When silencing CD44, 4Fh2c and integrin β1, the stimulation of MMP1 decreased significantly. Therefore, the hypothesis that extracellular gal-3 stimulates the MMP1 expression through its internalization is denied. Since if the internalization mechanism does exist, silence of one of these ligands would not affect the MMP1 stimulation by gal-3. Here it is worthy to mention that integrin β5 seems to restrain the MMP1 expression inherently. Indeed, we showed in our study that silencing of integrin β5 induced the expression of MMP1, and gal-3 reinforced greatly the induction, suggesting a direct recognition of this protein between cell matrix and the regulation of MMP1. However, as described above，silencing of integrin β5 completely counteracts the stimulation of leptin expression by gal-3. This suggests that even in the same cell type, integrin β5 plays different roles on different gene expression.

Surprisingly, when those ligands were silenced, the activation of P38 MAPK and

PI3K/AKT signaling pathway is not influenced as anticipated. Considering the short time needed to switch them on by Gal-3, and the failure to identify the accurate activated time point, maybe a future work should be conducted to precise the activated point for each signaling pathway rather than determine that at the same time.

It has been demonstrated that in osteoblasts, cytochrome p450 27B1 (CYP27B1) encodes the expression of 25-Hydroxyvitamin D_3 1-alpha-hydroxylase, which transfers $VitD_3$ to its activated form in the inner mitochondrial membrane. This activated form of $VitD_3$ binds to its receptor and regulates calcium metabolism, which is important for mineralization. We have investigated the effects of gal-3 on this enzyme and obtained some interesting results. It seems that gal-3 stimulates the expression of CYP27B1 under normoxia while inhibits that under hypoxia. With the inhibitors of signaling pathways activated by gal-3, we found that though the same signaling pathways are involved under normoxia and hypoxia, they acts totally differently. It is should be noted that the expression of CYP27B1 is relatively weak in human osteoarthritic osteoblasts, as through qPCR we observed that the CT values are above 30. Therefore, the results obtained maybe not exact because of the limitation in detection sensitivity.

As mentioned above, OCN is an important metabolic marker of osteoblasts. This protein is characterized by γ-carboxyglutamic acid residues, whose synthesis occurs post-translationly. This process depends on γ-glutamyl carboxylase (GGCX), Vitamin K, which is proposed to prevent the joint from OA progression due to its potential bone and cartilage effects[75,76], served as a cofactor. Our work demonstrated that gal-3 stimulates the expression of GGCX under normoxia and hypoxia. The regulation mainly passes through PI3K under normoxia and via PI3K and p38 MAPK under hypoxia. ERK is mildly involved in these different oxygen tensions. However, attention should be paid that we measured the gene expression rather than enzyme activities in our study, which renders our results validated by a single technique. Moreover, according to our results, gal-3 seems to favor the γ-carboxylation, which is needed for intact OCN and then to ensure its role. This contradicts with previous researches, since a deleterious role has been endowed for gal-3 in bone metabolism. Therefore, we suspect that it may exist a retro control mechanism in osteoblasts to repair the bone in case of the recruitment of inflammatory phase.

Overall, our study indicates that gal-3 modifies the osteoblast phenotype and triggers

this cell to produce MMP1, which is a degradative enzyme directed against bone collagen. Therefore, gal-3 seems to have a bony deleterious role.

CHAPTER V

GENERAL CONCLUSION

GENERAL CONCLUSION

The objective is to investigate the osteoblast phenotype alterations during the OA process, to detect the effects of gal-3 on osteoblasts under normoxia and hypoxia, to explore the mechanism of phenotype modifications and finally to identify by which ligands gal-3 carry out these effects.

In summary, we have identified 2 populations of osteoarthritic osteoblasts based on the osteocalcin expression under the control of $VitD_3$, which is reflected by different gene expression related to Wnt/β-catenin signaling pathway (Y.Hu *et al*., 2015).

Under normoxia, the expression of MMP 1, an interstitial collagenase responsible for type I collagen degradation, is induced by gal-3. This induction is regulated by 4F2hc, partly to CD44 and integrin β1 and via p38 MAPK and PI3K/AKT signaling pathways. Under hypoxia, the MMP 1 expression is similar although CD44 seems not to be involved.

Leptin, an adipokine implicated in the regulation of skeletal biology and that contributes to OA, is also induced by gal-3 with the involvement of ERK1/2, p38 MAPK and PI3-K/AKT pathways and with 4F2hc and integrin β5 as well. (Y.Hu *et al*., in preparation).

Overall, our study indicates that gal-3 modifies the osteoblast phenotype and triggers this cell to produce MMP1, which is a degradative enzyme directed against bone collagen. In regards to the literature and our data in this thesis, which demonstrated that extracellular gal-3 has deleterious roles in joint by acting on multiple targets, it would be interesting to counteract the gal-3 effects. On the other hand, it is also well known that intracellular gal-3 is benefit for chondrocyte biology and therefore this effect should be preserved. Therefore, one way to prevent extracellular gal-3 roles would be prevent its binding to the several glycosylated structures on cell membrane. To reach

this aim, gal-3 antagonists having a higher affinity for these structures than gal-3 or able to be distributed with a high bioavailability in the body must be developed.

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Résumé

La cellule principale de l'os sous-chondral est l'ostéoblaste qui joue un rôle central dans la production qualitative et quantitative de la matrice ostéoïde. Plusieurs études montrent que le collagène de type I et la phosphatase alcaline sont des marqueurs précoces de la différenciation des ostéoblastes tandis que l'ostéocalcine (OCN) et la minéralisation sont des marqueurs des stades tardifs de cette différenciation. L'os sous-chondral est le site actif de nombreux changements morphologiques qui peuvent être différents au cours de l'arthrose (OA) mais qui sont partie prenante du processus pathologique. Ces changements consistent en une formation de la matrice osseuse importante associée une inhibition de la minéralisation. Ces phénomènes peuvent être reliés aux changements phénotypiques des ostéoblastes.
La galectine-3 (gal-3) est un facteur inflammatoire qui a été détecté dans le tissu synovial et dans le liquide synovial lors d'inflammation d'OA. Des études antérieures ont montré que la gal-3 participait à la destruction du cartilage et inhibait fortement la production d'OCN par les ostéoblastes OA. Ces faits ont permis de suggérer que la gal-3 pouvait participer soit à l'initiation soit à la progression de l'arthrose. Peu d'études ont globalement été réalisées sur le rôle de la galectine-3 dans l'arthrose et encore moins sur le rôle de gal-3 sur les altérations de l'os sous-chondral.
Dans ce contexte, ce travail de thèse a consisté à caractériser les ostéoblastes arthrosiques, puis investigué la modulation du phénotype des ostéoblastes arthrosiques par gal-3 en et enfin identifier les mécanismes cellulaires impliqués.

D'une part, nous avons identifié deux populations ostéoblastes OA grâce à l'expression d'OCN. Dans les conditions basales, ces deux populations expriment de façon différentielle le TGF-β1, Wnt5b et DKK2, ce qui suggère une différenciation et un phénotype hétérogènes des ostéoblastes chez les patients OA. D'autre part, nous confirmons le rôle délétère de la gal-3 dans l'articulation lors d'inflammation puisqu'elle stimule la production de collagénase 1 impliquée dans la dégradation osseuse. De plus, elle accentue la perturbation phénotypique des ostéoblastes qui produisent plus de leptine lors d'épisodes hypoxiques. Bien que plusieurs ligands membranaires puissent médier les effets de gal-3, 4F2hc semble jouer un rôle récurrent.

Mots-clés : Galectine-3, arthrose, collagénase 1, leptine, ligands de galectine-3.

Abstract

Osteoblasts are the main cells in subchondral bone (SCB), which are responsible for the bone matrix production. Their differentiation can be evaluated by type I collagen and alkaline phosphatase in the early stage and by osteocalcin (OCN) and mineralization in the late stage. Alterations of SCB are essential episodes of osteoarthritis (OA) and are represented by a significant bone formation accompanied with abnormal hypomineralization. These changes in SCB are related to phenotypic modifications of osteoblasts.

Galectin-3 (Gal-3) is an inflammatory factor markedly detected in the synovial tissue and synovial fluid during OA inflammation. Previous studies have demonstrated that gal-3 was deleterious for cartilage and inhibited the production of OCN in OA osteoblasts. These findings suggest that gal-3 can participate in either the initiation or progression of osteoarthritis. So far, a few studies have been conducted to explore the role of Gal-3 in OA and particularly related to SCB.

In this context, the thesis has consisted to characterize OA osteoblasts, to investigate the modulation of the OA osteoblast phenotype by gal-3 and finally to identify the involved cellular mechanisms.

We have identified two populations of OA osteoblasts according to the OCN expression. Under basal conditions, these two populations express TGF-β1, Wnt5b and DKK2 differentially, suggesting various differentiation and heterogeneous phenotype of osteoblasts in OA patients. Moreover, we confirmed the deleterious role of gal-3 in the joint during inflammation since it stimulates the production of collagenase 1 involved in bone degradation. In addition, it emphasizes the disruption of phenotypic osteoblasts by producing more leptin during hypoxic episodes. Although several membrane ligands can mediate the effects of gal-3, 4F2hc seems to play a prominent role.

Keywords: Galectin-3, osteoarthritis, collagenase 1, leptin, galectin-3 ligands.