Characterization of the osteoarthritic joint microenvironment

Impact

Osteoarthritis (OA) is a debilitating joint disease with severe socio-economic consequences that globally affect 7% of the population[1]. OA is considered a multifactorial disease with common risk factors, such as age, sex, joint injury, obesity, genetic predispositions and mechanical stresses[2]. The onset of the disease is often unknown and many distinct clinical phenotypes (e.g. inflammatory, metabolic and mechanical overload) have been identified[3]. While patient numbers are growing, only few therapies are available. Currently, pharmacological treatments are limited to symptomatic pain relief medication. A major challenge to develop effective OA disease-modifying drugs is the heterogeneity of the disease. Therefore, future diagnostics-guided precision medicine might offer better treatment options. Both medical imaging and biomolecular techniques are expected to be required to guide this future decision-making[3].

The cell-based reporter gene assay is an attractive biomolecular technique that might contribute to both diagnostics and drug development for OA. Disease-associated genes can be rapidly screened using these reporter genes, as exemplified by the IL6 and WISP1 promoter reporter bioassays in **Chapter 3**. In addition, **Chapters 2 and 4-6** highlighted transcription factor response element-driven reporter genes as valuable tools for monitoring cellular signaling in response to a variety of stimuli (e.g. body fluids, proteins and small molecules). Moreover, disease-related pathways can be screened for potential drug entities (e.g. kinase inhibitors) using these reporters (**Chapter 4/5**).

Although mechanistic insight into OA initiation and progression is emerging, several underlying molecular processes remain to be understood. By example, only a handful of studies focused on investigating the underlying mechanisms promoting cartilage fibrosis[4-6]. In this thesis, synovial fluid derived from end-stage OA patients was used as an OA-relevant stimulus for in vitro culture of primary chondrocytes. **Chapter 4** highlighted the overlapping gene expression signatures between our OA synovial fluid-cultured chondrocytes and fibro-chondrocytes residing in OA cartilage of patients[7]. Therefore, we postulate that OA synovial fluid can be used as an in vitro model to study chondrocyte fibrosis. Moreover, future work can be extended to ex

vivo cartilage culture with OA synovial fluid to simulate an environment even closer to the in vivo situation. The use of both models can have impact for pre-clinical testing of potential therapeutics in OA.

One of the hallmarks of OA development is the changing joint microenvironment, with a central role for synovial fluid[8-10]. In **Chapter 5**, we characterized the impact of OA synovial fluid on chondrocyte signaling and its associated phenotypic changes. In direct comparison with non-OA synovial fluid, OA synovial fluid promoted sustained chondrocyte proliferation, accelerated dedifferentiation, fibrosis, inflammation and matrix degradation. Characterization of OA-specific signaling changes and pathway-phenotype relationships yielded many molecular targets to potentially impact OA-related processes. In **Chapter 4**, it is highlighted that growth factor-enriched OA synovial fluid sustains proliferation via EGF receptor signaling. Interestingly, enhanced proliferation has been considered a cartilage protective mechanism by increasing the number of chondrocytes in the superficial layer[11]. Recently, it has been shown that inhibition of EGF signaling accelerates OA development, while TGFα-induced EGFR signaling can prevent it[12]. Although activating EGFR signaling in the early stages of OA appeared effective, further research is required to establish its effectiveness in later stages when the superficial cartilage zone is disrupted. The acquired proliferative potential of chondrocytes upon OA synovial fluid exposure occurred simultaneously with rapid dedifferentiation (**Chapter 4**). Loss of the chondrogenic phenotype is one of the processes that is associated with OA progression and compromises the cartilage extracellular matrix. Interestingly, OA synovial fluid-induced loss of chondrocyte phenotype was rescued by ERK inhibition (e.g. SCH772984), whereas the proliferation rate remained unaffected (**Chapter 4/5**). Targeting specific pathways (e.g. MAPK/ERK) to counteract OA, or fine-tune other treatments, might be an attractive approach. Success of such strategy can greatly depend on the specificity of the selected kinase inhibitor. Alternative to conventional ATP-mimicking inhibitors with many off-targets, highly selective non-canonical binding mode inhibitors should be the preferred choice[13]. Other pharmaceutical strategies might include chondrogenesis-enhancing molecules, such as teriparatide (PTH^{1-34}), sprifermin (rhFGF18) or Kartogenin, to maintain the chondrogenic phenotype[14-16]. Moreover, dual treatments might be

considered to effectively stimulate proliferation and prevent loss of phenotype. Other typical OA-related processes, fibrosis and inflammation, were provoked by an OA synovial fluid microenvironment. Our in vitro inhibitor screen uncovered mainly JNK (SP600125), PI_3K (LY294002) and classical PKCs (cPKC; Gö6976) as key drivers of chondrocyte fibrosis (**Chapter 5**). Currently, clinical trials are conducted using improved JNK (i.e. CC-930) and PI_3K (i.e. Omipalisib) inhibitors to treat idiopathic pulmonary fibrosis[17,18]. These highly selective JNK and PI_3K inhibitors can be interesting candidates for chondrocyte fibrosis treatment. Alternative strategies to counteract chondrocyte fibrosis may include triggering the anti-fibrotic pathways, like cAMP (e.g. Butaprost) and PPARγ (e.g. Rosiglitazone)[19]. Signaling protein profiling of OA synovial fluid revealed increased levels of various pro-fibrotic chemokines (e.g. CCL24) that could be antagonized to reduce chondrocyte fibrosis (**Chapter 5**)[20]. The anti-fibrotic effect of the proposed pharmacological interventions may not be limited to chondrocytes, but could serve as potential therapeutics for OA-related synovial fibrosis as well[21]. In **Chapter 5**, we highlighted the increased inflammatory profile of the OA joint microenvironment. Many inflammatory proteins, including cytokines, chemokines and damage-associated molecular patterns (DAMPs), were upregulated specifically in OA synovial fluid. In accordance, secretomes of OA joint tissues (i.e. cartilage, meniscus, infrapatellar fat pad and synovium) revealed activation of inflammatory signaling routes in chondrocytes as well. Compared to non-OA, OA synovial fluid induced a more pronounced NFκB response that provoked upregulation of several chemokine genes. Interestingly, pharmacological inhibition of cPKC with Gö6976 abolished both OA synovial fluid-induced NFκB signaling and chemokine responses. This highlights a major role of cPKC for OA synovial fluid-induced inflammatory responses, where previous research primarily focused on cPKC-independent inflammatory signaling, such as interleukins[22]. OA synovial fluid is greatly enriched in chemokine receptor (e.g. CCL24, CXCL12 and CXCL13) and Toll-like Receptor 4 (e.g. S100A10/12 and SAA1) agonists that are known to activate cPKC signaling[23,24]. Therefore, both receptor antagonism and cPKC inhibition hold promise for treatment against OA-related inflammation. Collectively, our characterization of pathway-dependent chondrocyte phenotypic changes in an OA microenvironment

serves as fundamental knowledge for future studies focusing on the development of OA therapeutics.

Future therapeutic decision-making may depend on both the OA stage as well as the patient-specific phenotypes. While many different clinical phenotypes have been reported, the corresponding underlying molecular disease processes (i.e. endotypes) remain to be elucidated[3]. The ultimate challenge is to develop diagnostics that detect molecular disease changes before clinical phenotypes arise. For this purpose, biomolecular tools will play a vital role. In the last two decades, numerous studies identified potential stratification biomarkers in synovial fluid, but none have so far proven sufficiently discriminating to be applied for clinical diagnostics. To overcome this challenge, we aimed to develop an alternative cell-based approach that can integrate the molecular signaling complexity of synovial fluid (**Chapter 6**). A set of six transcription factor response element-driven reporter genes was selected for a screening of 160 end-stage OA synovial fluids. The OA synovial fluids classified into two major subgroups based on the integrated signaling responses. Interestingly, five out of six (i.e. NFκB-RE, CRE, SRE, SRF-RE and AP1-RE) reporter genes responded in the same manner, suggesting that similar upstream activators or networks could regulate these. In contrast, one reporter gene (i.e. SIE) exhibited opposing response patterns. This classification resolution might be further enhanced in the future by adding additional reporter genes that inform on distinct molecular signaling routes. For future clinical application for biomolecular OA stratification, a single readout system of five reporter genes with improved pathway resolution could be generated by coupling response elements to unique luciferase variants as described recently[25]. While, **Chapter 6** highlights the potential use of these reporter genes to stratify OA, future research will be required to validate the discriminative capacity in the early stages of OA development. Moreover, the biological consequence of the identified OA subgroups needs further investigation to accurately characterize these OA synovial fluid endotypes. An additional OA clinical diagnostic strategy might be to identify stratification biomarkers within the cell-based reporter system pre-defined OA synovial fluid endotypes. Either diagnostic approaches might aid future OA treatment decision-making by providing an additional layer of patient-specific information at the molecular level. This molecular endotyping might fill the diagnostic

gap that explains the disease processes linked to a certain clinical phenotype as clinically determined by imaging techniques. Where the one-treatment-fits-all remains ineffective[3], endotype-guided treatment selection might improve therapeutic efficacy. A first step to explore the impact of stratification medicine can be assessed in clinical trials by selectively recruiting OA patients based on endotypes that are likely to benefit from the investigated drug. By example, the endotypes (i.e. MAPK/ERK and STAT3 signaling) identified in this thesis might require different treatment strategies (e.g. ERK inhibitor; SCH772984 or IL6/STAT3 antagonist; Tocilizumab), as shown in Figure 1. In case of multiple underlying disease processes, multi-drug therapy can potentially be considered. Depending on whether the OA endotypes are initiated by local or systemic triggers, drugs can be administered accordingly, such as intra-articular injection or orally. Another critical factor in treatment success might be the timing of diagnosis and start of treatment. One can envision that if the disease onset is diagnosed early on, pharmacological strategies might have higher success rates. Therefore, future research is required to establish a threshold in which the OA phase is considered reversible and treatable with pharmaceutics or irreversible and surgical interventions (e.g. implants, joint distraction and total knee arthroplasty) might be the best solution. In the light of early diagnosis, individuals with known high-risk OA co-morbidities (e.g. diabetes and obesity) could be screened regularly at older ages. Taken together, the need for both OA molecular diagnostics and pharmaceutical therapies for OA is high, where potentially the one might not succeed without the other. This thesis lies a ground work for a novel approach for molecular diagnostics and highlights several important OA disease mechanisms that can be explored for pharmaceutical interventions.

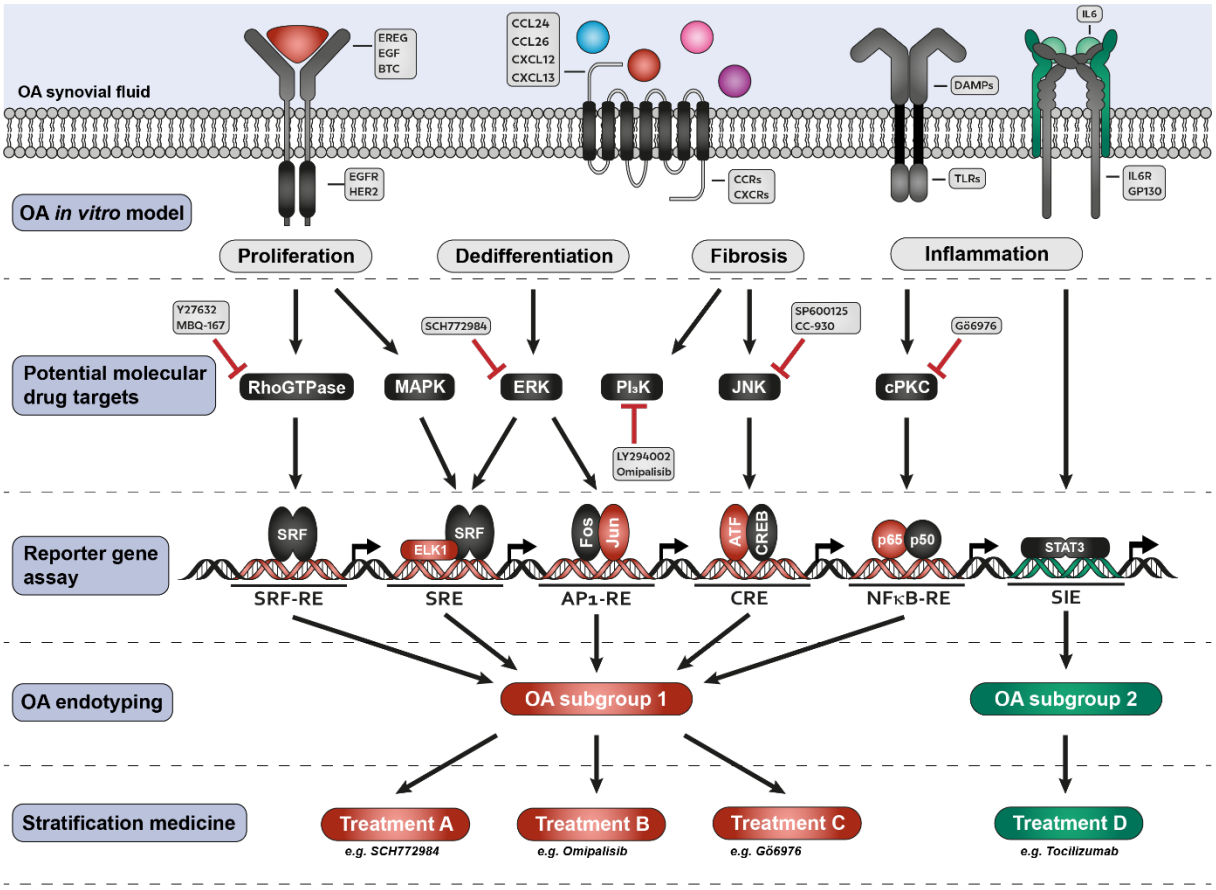

Figure 1: Summary of key findings. OA synovial fluid as simulated OA microenvironment promotes chondrocyte proliferation, dedifferentiation, fibrosis and inflammation. Interesting molecular signaling drug targets include ERK, PI_3K, JNK and classical PKC. Reporter gene assays serve as valuable tools for endotyping OA synovial fluid as well as studying pharmacological pathway modulation. Two major OA subtype were identified based on synovial fluid provoked signaling patterns. Distinct OA subgroups might be treated with endotype-specific therapeutics.

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