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Modulation of the canonical Wnt signaling pathway in bone and cartilage

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Chapter 7

Summary, conclusions, directions for future research

SUMMARY AND CONCLUSIONS

The interaction of Wnt proteins with their receptors and identification of Wnt signaling antagonists have been the foci of many studies and review articles (1-3). These studies show that canonical Wnt signaling is a very complex biological pathway, consisting of a large number of genes, receptors, agonists and antagonists, which bind to each other in many different combinations to elicit a large number of responses in a variety of cells and tissues. Many studies have now identified that the Wnt signaling pathway has a crucial role in growth plate biology, bone formation and skeletal remodeling (4-13). Most of these data were based on observations made in animal models in which the main effector of the canonical Wnt signaling, β-catenin, is either forcedly expressed or inactivated. Still at the time of the start of our studies, the cellular mechanisms by which canonical Wnt signaling controls skeletal development and maintenance via wild-type β-catenin were largely unknown. No reports were available on the role of Apc in regulation of skeletal precursor cell (SPC) differentiation via control of β-catenin. In addition, no studies had been reported on the possible beneficial effect of human *APC* mutations on bone mass, while only a few reports were published investigating the function of Gsk3β in cartilage maintenance (14). The experiments described in this thesis were designed in order to improve our understanding of the role of proteins acting up-stream of β-catenin during skeletal development and maintenance. Our findings and conclusions indicate that Apc and GSK3β, the two master intracellular regulators of the levels of transduced canonical Wnt signaling, fulfill major roles in the regulation of chondrocyte and osteoblast biology and pathology by adjusting the protein level of β-catenin.

The *Cre*/loxP system is a tool for tissue-specific knockout of those genes which cannot be investigated in differentiated tissues because of their early embryonic lethality using conventional knockouts (15). We initially used this genetic system to investigate whether Apc is involved in lineage commitment of SPCs by generating conditional knockout mice lacking functional Apc in *Col2a1*-expressing cells (16). Our data indicate that the *Col2a1* promoter is suitable for this study, since *Cre*-mediated recombination starts very early (E9.5) in SPCs that have not yet committed to the chondrogenic or the osteogenic lineage, consistent with previous findings in other Col2a1-*Cre* lines (17;18). Lack of functional Apc resulted in a pleiotropic skeletal cell phenotype. In the vast majority of SPCs, loss of functional Apc led to a strongly increased β-catenin level, resulting in the formation of an undifferentiated mesenchymal cell, with no differentiation potential for both osteogenic and chondrogenic lineages. When the inhibitory effect of a strongly increased β-catenin level in the skeletal precursors was reduced, highly active osteoblasts arose. Strong repression of β-catenin in these precursors was required for chondrogenesis. Our data presented in **chapter 2** indicate that a tight Apc-mediated control of β-catenin levels is essential for differentiation of skeletal precursors as well as for the maintenance of a chondrocytic phenotype in a spatio-temporal regulated manner.

We continued our expedition in unraveling the role of Apc in skeletal development by generating compound conditional Col2a1-*Cre*;*Apc*$^{15lox/1638N}$ and Col2a1-*Cre*;*Apc*$^{15lox/1572T}$ mutant embryos. Distinct levels of Wnt/β-catenin signaling in the two types of embryos differentially affected the chondrogenic and osteogenic differentiation of SPCs. Relatively high levels of β-catenin in the SPCs of Col2a1-*Cre*;*Apc*$^{15lox/1638N}$ embryos led to a complete blockade of both chondrocyte and osteoblast formation, while intermediate levels of β-catenin in the SPC of Col2a1-*Cre*;*Apc*$^{15lox/1572T}$ embryos led to regional-specific modulation of SPC differentiation. Whereas sclerotomal SPCs expressing *Apc*$^{\Delta 15/1572T}$ failed to differentiate into chondrocytes and osteoblasts, in the inferior mandible and long bones these cells differentiated into highly active osteoblasts. Gradients of soluble growth factors regulate many developmental processes such as tissue organization, patterning and segmentation (19). By generating conditional compound Col2a1-*Cre*;*Apc*$^{15lox/1638N}$ and Col2a1-*Cre*;*Apc*$^{15lox/1572T}$ transgenic mice, we prove in **chapter 3** that different *Apc* mutations resulting in different levels of canonical Wnt signaling have distinct effects on the differentiation of SPC. This implies that a tight regulation of the dosage of functional Apc is directive for the lineage commitment of SPC via modulation of β-catenin.

To be able to mechanistically analyze the role of Apc in the regulation of SPC differentiation into chondrocytes and osteoblasts, we next knocked down the mouse *Apc* gene using RNA interference (RNAi) in the murine mesenchymal stem cell-like KS483 cell line (20). This cell line has SPC-like characteristics, since it can form osteoblasts, chondrocytes, and adipocytes (21). Our results indicated that Apc is required for proliferation, suppression of apoptosis and for differentiation of murine mesenchymal stem cell-like KS483 cells into the osteogenic, chondrogenic and adipogenic lineage. *Apc* knockdown led to up-regulation not only of the Wnt/β-catenin, but also of the BMP signaling pathway, further sustaining the interaction of these biological routes during various steps of SPC differentiation. Interestingly, the inhibitory effects of *Apc* knockdown on osteogenic differentiation could be rescued by high levels of exogenous BMP-7. It is noteworthy to mention that we obtained similar results by using 2 different shRNA sequences targeting *Apc*, while stable transfection of the respective control mutant shRNA plasmids (containing 2 nucleotide mismatches) did not alter the proliferation, survival and differentiation capacity of KS483 cells. This clearly indicates that our results were the consequence of a bona-fide and specific siRNA effect lowering wild-type Apc expression. Partial rescue of the Wnt-responsive BAT-Luc reporter activity by transient transfection of a human *APC* expression vector further confirmed these observations. Our approach described in **chapter 4** provides a valuable model demonstrating that levels of functional Apc must be tightly controlled for proper modulation of the transcriptionally active β-catenin and BMP-signaling dosage required for multilineage SPC-differentiation *in vitro*.

To further analyze the role of APC in the skeleton and thereby to complete our *in vivo* and *in vitro* mouse studies, we next performed the first cross-sectional, population-based study documenting a detailed assessment of bone and mineral metabolism in familial adenomatous polyposis (FAP) patients carrying heterozygous mutations in the *APC* gene (22). Our results indicated that FAP patients display a statistically significantly higher mean BMD in comparison with age and sex matched controls. In our

study population mean P1NP (marker of bone formation) and mean β-CTX (marker of bone resorption) concentrations were within the normal ranges and were significantly positively correlated. Furthermore, both these markers were positively correlated with bone mineral density (BMD). Our data described in **chapter 5** suggest a state of "controlled" activation of the Wnt signalling pathway in heterozygous carriers of *APC* mutations, most likely due to upregulation of β-catenin resulting in a higher than normal BMD. Our findings in FAP patients are sustained by data reported in relatives of patients with sclerosteosis, who carry heterozygous mutations in the *SOST* gene, another negative regulator of the canonical Wnt signaling pathway (23). Findings from these two human genetic models may be exploited in the identification of potentially attractive therapeutic targets in the treatment of osteoporosis.

Studies reported so far indicate that Gsk3β activity is required for both chondrocyte and osteoblast differentiation and thus for endochondral bone development (24). However, no data was available regarding the role of Gsk3β in maintenance of the chondrocytic phenotype. To investigate this we finally treated chondrocytes *ex vivo* and *in vivo* with GIN, a selective GSK3β inhibitor (25). Gsk3β, by controlling the canonical Wnt signaling pathway, was critical for maintenance of the chondrocytic phenotype. Inhibition of Gsk3β in fetal mouse metatarsals led to loss of cartilage markers expression, induced matrix degradation by stimulating the expression of Mmps, inhibited chondrocyte proliferation and, most likely as a consequence of these effects, induced chondrocyte apoptosis. In addition, transient inhibition of Gsk3β, following three intra-articular injections of GIN in rat knees during one week was associated with the appearance of osteoarthritis (OA)-like features six weeks later. In agreement with our results, recent findings suggest that upregulation of β-catenin through induction of proteasomal degradation of Gsk3β in chondrocytes initiates early events of OA, while inhibition of Gsk3β may block chondrogenesis (26;27). Abnormally regulated GSK3β has been associated with many pathological conditions like Alzheimer's disease, mood disorders, diabetes and cancer (28). However, a direct link between GSK3β and the pathophysiology of OA has not yet been reported. Since in our experimental set-ups described in **chapter 6** the GIN-induced effects reflect some of the pathological findings normally seen in osteoarthritic chondrocytes, we speculate that Gsk3β plays a role in the pathophysiology of this degenerative cartilage disease as well, most likely by regulating the levels of β-catenin.

DIRECTIONS FOR FUTURE RESEARCH

Our *in vivo* results obtained in the conditional *Apc* mutant mice indicate that a tight regulation of the levels of β-catenin by Apc is crucial during skeletal development. An important drawback of our studies is that we cannot exactly indicate the timeslot when the regulation of SPC differentiation by Apc begins during embryogenesis and how this expands in subsequent developmental stages. Developing an inducible conditional mouse line carrying both a tissue- and time-specific Apc inactivation will enable us to more precisely unravel the role of Apc during distinct steps of SPC differentiation

into chondrocytes and osteoblasts. Nevertheless, performing *in vitro* differentiation experiments, for instance by using mouse embryonic fibroblasts from *Apc*$^{+/15lox}$, *Apc*$^{15lox/15lox}$, *Apc*$^{+/1638N}$, *Apc*$^{+/1572T}$, *Apc*$^{15lox/1638N}$ and *Apc*$^{15lox/1572T}$ mice infected with *Cre*-lentiviruses could provide a more quantifiable approach to investigate the regulation of SPC differentiation by Apc.

In our cross-sectional, population-based study, we demonstrated that FAP patients display a significantly higher than normal mean BMD compared to age- and sex-matched healthy controls in the presence of a balanced bone turnover. Whether increased bone mass accrual is sustained over the years, and whether this higher BMD than normal may reduce age-related fracture risk remains to be established by long-term follow-up studies in FAP patients.

Our *in vivo* results from the GIN-treated rat knees suggest that Gsk3β inhibition can induce cartilage degeneration of rat articular cartilage after 3 intra-articular injections of GIN with a two days interval. It will be interesting to test whether the mild phenotypic effects resembling early stages of OA would result in more exaggerated effects in a longer follow-up time. Nevertheless our results indicate that β-catenin upregulation in cartilage secondary to Gsk3β inhibition is sufficient to induce OA-like features *in vivo*, thereby providing a valid model for the study of OA. Whether pharmacological modulation of Gsk3β might represent a potential novel therapeutical approach for the management of OA remains to be elucidated.