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Dickkopf-1 Induces Migration in Fibroblast-like Synoviocytes

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by

Bryan V. Dieffenbach

Committee in charge:

Professor Mary P. Corr, Chair
Professor Stephen Hedrick, Co-Chair
Professor Elina Zuniga

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Co-Chair

Chair

University of California, San Diego

2010

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VITA

- 2009 Bachelor of Science in Biology, University of California,
San Diego
- 2009-2010 Teaching Assistant, Department of Biological Science
University of California, San Diego
- 2010 Master of Science in Biology, University of California, San
Diego

ABSTRACT OF THE THESIS

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Bryan V. Dieffenbach

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Professor Mary P. Corr, Chair

Professor Stephen Hedrick, Co-Chair

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting joint tissues. Disease is characterized by synovial hyperproliferation and joint invasion, severe inflammatory cell influx and matrix destruction. Serum levels of the wnt antagonist, Dickkopf-1, are increased in diseased patients and have been described to affect bone homeostasis in affected joints. Here we introduce an additional role for Dickkopf-1, the induction of migration in fibroblast-like synoviocytes (FLS) *in vitro*.

Our results show that Wnt5a and surprisingly, DKK1 induce migration in wound edge C57Bl/6 FLS. Wnt5a has previously been demonstrated to induce migration in a c-jun N-terminal kinase (JNK) dependent manner whereas DKK1 has been implicated in both induction and inhibition of migratory behavior. Migrating FLS exhibit activation of JNK1 and JNK2, and the absence of either isoform limits

the induction of synoviocyte migration, indicating that JNK is required for FLS migration. However, Dickkopf-1 treatment of JNK1 deficient FLS was able to induce migration in wound edge cells, while JNK2 deficient FLS were refractory. These findings suggest that the Wnt signaling pathways contribute to migration in FLS *in vitro*. Elevated signaling through Wnt-associated molecules may account for the aberrant migratory behavior of synovial cells in rheumatoid arthritis patients.

1. Introduction

1.1. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disorder whose onset has yet to be fully determined. The characteristic phenotype of disease is chronic synovial inflammation and structural degradation of the distal joints. Patients tend to exhibit increase serum concentrations of rheumatoid factor (immunoglobulins that bind the Fc region of IgG) and other key inflammatory proteins/cytokines including interleukin (IL)- 1β , Tumor Necrosis Factor (TNF) α and interferon (IFN) γ (Emery, 2002). Studies of the prevalence of RA reveal that the risk of development increases with age. The disease is prevalent in nearly 1% of the US population making it the most common autoimmune disease in the nation (Lee, Beck et al. 2008). Within these populations, women appear to be affected nearly two times more often than men, indicating that sex plays a role in the development of inflammatory arthritis (Mitchell 1985).

The mechanism for development of RA has yet to be elucidated, but has been considered to be the result of interrelationships between adaptive immunity, innate immunity and genetics. Previous studies have shown that some patients with RA exhibit elevated serum concentrations of antibodies reactive toward foreign antigens with homologous regions to those of self-proteins. As a result, through molecular mimicry, an immune response which was initially meant to target foreign antigen can be perpetuated as it begins to target self-protein (Moreland and Koopman 1991). In addition to these findings, diagnostic tests have been studied based on the prevalence of

autoantibodies in affected patients' serum. One such autoantibody is anti-cyclic citrullinated peptide (CCP) autoantibodies and appears to be accurate in predicting disease development. To date, the role of citrullinated peptides in the pathogenesis has not been determined (Lee, Beck et al. 2008).

Genetic risk factors for RA disease development have been hypothesized and may contribute to disease pathogenesis and the production of rheumatoid factor (autoantibody prototypes that react with the Fc region of IgG molecules). Associations between RA disease development and certain alleles at the human leukocyte antigen (HLA) locus HLA-DR have been shown to contribute to disease pathogenesis. The alleles DRB1*0401, *0404, *0405, *0408, *1402, *0101, *0102, and *1001 all show common variations at the third hypervariable region of the DRB chain. Interestingly, other alleles that do not show an association with RA lack the variant motif in the third hypervariable region of the DRB chain (Gregersen, Silver et al. 1987; Perdriger, Guggenbuhl et al. 1996).

Mutations in the genes encoding kappa light chains of rheumatoid factors correlate with an increased risk of RA development. It was shown the mutations at these gene loci are novel predictors of RA susceptibility due to the lack of heterogeneity amongst these genes (Olsen and Chen 1991). Using genome-wide association techniques on patients affected with rheumatoid arthritis, several other genes have been hypothesized to contribute to disease susceptibility. protein tyrosine phosphatase (PPTN) 22, expressed mainly in lymphocytes is necessary for normal lymphocyte signaling through the TCR/BCR. Variants at this locus seem to develop RA more often suggesting that altered TCR/BCR signaling contributes the disease phenotype. TNF receptor associated factor

(TRAF)1, variants also seem to have increased disease prevalence suggesting that altered TNF signaling plays a role in RA as well (Yamamoto and Yamada 2007). The overall findings to date suggest that RA development and perpetuation is dependent on the summative effects of multiple disease promoting events.

The pathologic processes that occur during the course of RA include contributions from both adaptive and innate immune responses from a variety of cell types. Adaptive immune cells are present in affected RA joints. Among the mononuclear cells present, activated B-cells and plasma cells secrete rheumatoid factor that have been described as potential contributors to disease severity and perpetuation, depending upon the secreted autoantibody specificity and target. Activated T cells, which are major regulators of immune response, have also been isolated from affected synovial tissue. Interestingly, few activated effector T cells are usually isolated from RA affected tissues but, treatments directed towards inhibition of T cell activation have shown to help ameliorate disease (Harris 1990). Studies on the role of T cells in the pathogenesis of arthritis have introduced a concept whereby superantigens may contribute to the generation and perpetuation of disease by activating large numbers of T cells in the affected area through nonspecific MHC binding. A definitive role for adaptive immune cells in rheumatoid arthritis has yet to be determined.

RA development is also dependent on the activity of various innate immune cell types. Neutrophil accumulation in the synovial fluid is common in RA. These immune cells are chemotactically attracted to the affected tissue by complement protein C5a, Leukotriene B4 and IL-8. Activated neutrophils secrete various proteinases, leukotrienes and prostaglandins that promote both joint destruction and the influx of other

inflammatory cells (Mitchell 1985). Macrophages have also been described as a major component of cell infiltrate into the rheumatoid joint. Activated macrophages release a number of inflammatory cytokines including IFN- γ , Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF), IL-2, IL-1 α , TNF α , and Transforming Growth Factor (TGF) β . These cytokines are capable of stimulating other cell types present in the affected joint and perpetuating the inflammatory response (Wilder, Lafyatis et al. 1990; Firestein 1991; Remmers, Sano et al. 1991).

Fibroblast-like Synoviocytes (FLS), the cells that line joints, contribute to innate immune protection. However, in RA they exhibit an unusual phenotype including sustained hypertrophy. These cells develop a partially transformed phenotype and become migratory and highly invasive, resulting in synovial pannus formation, extension of synovial cells into the joints tissues. Pannus formation contributes to joint destruction by production of matrixmetalloproteinase and an inhibitor of Receptor Activator for Nuclear Factor κ B Ligand (RANKL) expression which stimulates osteoclasts. Bone tissue homeostasis is also affected in RA and results in osteopenia of the affected joints. This is dictated by the effects of RANKL and various secreted proteins that downregulate osteoblast differentiation and increase osteoclastic activity.

1.2. Wnt Signaling

Wnt proteins are secreted lipid-modified proteins that bind to a large family of receptors. Wnt signaling has been attributed to a number of important roles including: development, differentiation, proliferation, cytoskeletal management, cell migration and

homeostasis. In order to fulfill these roles, Wnt proteins bind to a number of cellular receptors and activate many pathways.

Wnt/ β -Catenin signaling (canonical Wnt signaling) is among the most understood Wnt-induced pathway (Figure 1). In this pathway, Wnt binds to the frizzled receptor and low density lipoprotein (LDL)-receptor related protein (LRP) 5/6 complex to initiate downstream β -Catenin accumulation in the cytoplasm and subsequent translocation into the nucleus. In the absence of Wnt ligand, a “destruction complex” forms between Casein Kinase I (CK1), Glycogen Synthase Kinase (GSK) 3 β , Axin and Adenomatous Polyposis Coli (APC). This complex maintains a low cytoplasmic concentration of β -Catenin by sequestering and phosphorylating it at distinct N-terminal serine and threonine residues. The mechanism by which β -Catenin is labeled for degradation is initiated by a “priming event” in which CK I phosphorylates β -Catenin. This stimulates subsequent phosphorylation of the serine/threonine residues at the N-terminus by GSK3 β (Liu, Li et al. 2002). These signals recruit β -Transducin repeat containing protein (β -TrCP) to polyubiquitinate β -Catenin and initiate degradation at the proteasome.

The presence of extracellular Wnt ligand activates this pathway. This results in the phosphorylation of LRP5/6 by GSK3 β and CK1 followed by the recruitment of Axin and Dishevelled proteins to the membrane. The presence of Axin at the membrane inhibits destruction complex formation by diminishing the cytoplasmic Axin required to anchor the complex (Cadigan and Nusse 1997; Polakis 2000; Polakis 2002). This results in the accumulation of free cytoplasmic β -Catenin that can be translocated into the

nucleus. Nuclear β -Catenin interacts with LEF/TCF transcription factors and the complex can initiate expression of target genes. Notable genes induced by Wnt/ β -Catenin signaling are: c-Myc, Cyclin D1, Transcription Factor-1 (TCF), Peroxisome proliferative activated receptor-delta (PPAR), Matrixmetalloproteinase-3 (MMP), MMP7, Vascular endothelial growth factor (VEGF) and Axin2.

β -Catenin has also been described to interact with the cell-cell adhesion protein E-Cadherin. E-cadherin is a transmembrane protein whose cytoplasmic domain has been described to regulate cell-cell adhesion. Studies on cells containing mutations at the cytoplasmic domain of E-cadherin demonstrate decreased cell adhesions and suggest that the intracellular domain regulates these adhesions (Nagafuchi and Takeichi 1988). β -Catenin binds to the cytoplasmic domain of type I Cadherins, including E-Cadherin, and regulates structural organization and function within the cell (Gumbiner 2000; Jamora and Fuchs 2002). This interaction between β -Catenin and E-Cadherin is tightly regulated by the phosphorylation and dephosphorylation activity of receptor tyrosine kinases (RTKs) and transmembrane protein tyrosine phosphatases (TMPs), respectively. Activation of RTKs results in the phosphorylation of β -Catenin at tyrosine 654 and tyrosine 142 leading to dissociation of the protein from Cadherin and accumulation in the cytoplasm. This activity has been described to promote cell migration by decreasing cell-cell interactions (Lickert, Bauer et al. 2000; Bek and Kemler 2002; Lilien, Balsamo et al. 2002). Phosphorylation of tyrosine residues on β -Catenin has also been shown to induce endocytosis of the Cadherin complex in addition to β -Catenin dissociation (Le, Yap et al.

1999). TMPTP activity retains the non-phosphorylated form of β -Catenin and results in the maintenance of the β -Catenin/Cadherin complex.

Overall, the Cadherin pathway plays a role in β -Catenin homeostasis through tight regulation by tyrosine phosphatases and kinases. RTK activity is required for the dissociation of β -Catenin from the cell membrane complex and results in decreased cell-cell adhesion while increasing the concentration of cytoplasmic β -Catenin (Piedra, Miravet et al. 2003). This is supported by the observation that E-Cadherin repression results in increased β -Catenin signaling. TGF β has also been shown to inhibit E-Cadherin through SNAIL while also activating SMAD signaling. TGF β signaling activates expression of many β -Catenin inducible target genes which may reflect another pathway for expression of β -Catenin inducible genes (Li, Chen et al. 2006). These results suggest that Wnt/ β -Catenin signaling can be cross regulated by Wnt-independent pathways (Riese, Yu et al. 1997; Labbe, Letamendia et al. 2000; Nishita, Hashimoto et al. 2000).

Wnt signaling has also been described to regulate cytoskeletal homeostasis and migration through several β -Catenin-independent pathways, these pathways are considered to be “non-canonical”. Wnt5a has been shown to induce axonal guidance in neurons via atypical receptor related tyrosine kinase, Ryk, and modulate cell migration and proliferation through Receptor tyrosine kinase-like orphan receptor 2 (ROR2), both pathways are independent of canonical Wnt signaling (Liu, Shi et al. 2005). Through activation of ROR2, Wnt signaling has been suggested to induce cellular migration while inhibiting canonical signaling (Mikels and Nusse 2006). A possible mechanism of this

relates to an increase in c-jun terminal kinase (JNK) signaling as a result of Wnt5a and ROR2 interaction. Wnt5a/ROR2 signaling has demonstrated importance as a novel inducer of cell migration by causing downstream activation of JNK. The proposed mechanism by which this occurs was determined using chemical inhibitors of important cytoskeletal and signaling proteins. Small interfering RNA knockdown of Filamin A (FilA), an actin-binding protein involved in actin crosslinking, resulted in decreased JNK activation after stimulation with Wnt5a. This suggests that FilA mediates Wnt5a signaling (Yamamoto, Yoo et al. 2007). Subsequent experimentation showed that inhibition of the kinase PKC ζ , which is usually activated upon wounding, decreases JNK activation following Wnt5a treatment (Yamamoto, Yoo et al. 2007; Nomachi, Nishita et al. 2008). These findings are also supported by prior data showing that ROR2 receptor overexpression in cells results in increased filopodia formation (Nishita, Yoo et al. 2006). The collective findings indicate that Wnt5a contributes to cell migration and cytoskeletal homeostasis and that it likely acts through FilA and PKC ζ to activate c-jun terminal kinase (JNK).

Another β -Catenin-independent pathway is the Planar Cell Polarity (PCP) pathway. Although this pathway is less understood, it is believed that extracellular interactions with integrin receptors cause an intracellular cascade via cdc42, par, and GSK3 β . In astrocytes, a wound healing assay showed that cytoskeletal morphology is changed following elimination of cell-cell interactions (Etienne-Manneville and Hall 2001). Analysis showed that Cdc42, a Rho GTPase involved in the regulation of actin polymerization, is required for cell polarization. Cdc42 then interacts the PAR6 protein and complexes with PKC ζ to induce morphological changes in the wound edge cells.

These interactions could then determine cell polarity and direction of migration (Etienne-Manneville and Hall 2001; Etienne-Manneville and Hall 2003; Shi, Jan et al. 2003).

1.3. Wnt Protein Expression in Human Rheumatoid Arthritis

The synovial membrane exhibits an aberrant phenotype in RA. FLS exhibit hyperproliferation, similar to that of cancer cells, and subsequent migration onto joint tissues. Affected joints display elevated levels of enzymes involved in matrix destruction and proinflammatory cytokines and chemokines. Wnt pathways contribute to numerous cellular functions including cell cycle regulation, cell growth, survival, differentiation and adhesion. It has been hypothesized that the intracellular cascades induced by Wnt signaling could contribute to the activation of FLS in RA afflicted joints (Cadigan and Nusse 1997; Moon, Brown et al. 1997; Logan and Nusse 2004)). Studies by Logan et al. suggest that signaling mediated by Wnt/frizzled could contribute to numerous hyperproliferative disorders in adult tissues (Logan and Nusse 2004). Given that synovial cells display this aberrant phenotype in arthritic joints, it is possible that Wnt signaling could be responsible for the kinase activation and downstream transcriptional modifications characteristic in human RA.

Studies of rheumatoid synovia from joint replacement patients have shown that the expression of Wnt homologues Wnt5a, Wnt1 and frizzled 5 are elevated in affected joints when compared to osteoarthritic and/or normal tissues (Sen, Lauterbach et al. 2000). These results suggest a Wnt expression signature specific to RA. Cultured FLS

from RA patients also display persistent Wnt/frizzled gene expression lasting numerous passages *in vitro* (Sen, Reifert et al. 2002).

In vitro studies of wnt5a-transfected normal and osteoarthritis FLS resulted in a significant upregulation of the proinflammatory cytokines IL-6, IL-8 and IL-15 – all expressed in chronic rheumatoid arthritis tissues (Sen, Chamorro et al. 2001). It is hypothesized that the secretion of these inflammatory cytokines could contribute to the recruitment of leukocytes to the joint tissues and activate the hyperproliferative phenotype of rheumatoid FLS. In addition to these findings, Wnt1 signaling is constitutively active in RA fibroblast-like synoviocytes. This signaling induces downstream matrixmetalloproteinase 3 secretion and could explain the articular cartilage degradation characteristic in RA-affected joints.

1.4. Dickkopf-1

DKK-1 is a member of the Dickkopf family of proteins (DKK-1, DKK-2, DKK-3, and DKK-4). Structurally, DKKs consist of two cysteine-rich domains (one C-terminal, the other N-terminal) that are conserved across the four isoforms (Glinka, Wu et al. 1998; Krupnik, Sharp et al. 1999). Despite the structural homology between the Dickkopf family members, each has discrete functions on the cellular level. DKK-1 has gained notoriety as an inhibitor of the Wnt/ β -Catenin pathway (Figure 2). DKK-1 accomplishes this by binding to the LRP6 co-receptor, internalizing it in a clathrin-dependent manner. This disrupts LRP association with the Frizzled (Fz) receptor. As a result, the β -Catenin destruction complex remains functional and the cytoplasmic concentration of β -Catenin

is maintained at a low level and does not translocate into the nucleus to initiate transcription of target genes.

The DKK1 gene is induced in a number of ways. One way is a result of Wnt/ β -Catenin activation through Wnt3a interacting with Fz/LRP (Niida, Hiroko et al. 2004). Hence, the Wnt/ β -Catenin pathway can negatively regulate itself through the production of its own inhibitor following activation. DKK1 can also be induced by the inflammatory cytokine TNF α (Diarra, Stolina et al. 2007). TNF α binds to TNF Receptor1 and stimulates a cascade of kinase activities resulting in the secretion of various cytokines and inflammation. There is a strong correlation between increased serum DKK1 levels and rheumatoid arthritis which is supported by the observation that TNF α is upregulated in affected tissues. Human patients with RA exhibit nearly twice the serum concentration of DKK1 when compared to unaffected individuals. Proliferative synovia produce DKK1 (Diarra, Stolina et al. 2007). The increased serum DKK1 levels may result from activation of these cells by TNF α and/or Wnt3a in affected tissues.

Given that Wnt/ β -Catenin pathway has been associated with growth and development, DKK-1 acts as a strong regulator of these processes. It has been shown that DKK-1 is instrumental in head and brain development as well as cardiac and limb development (Marvin, Di Rocco et al. 2001; Mukhopadhyay, Shtrom et al. 2001; Schneider and Mercola 2001). As discussed above, DKK1 has also been shown to regulate bone remodeling. It has been suggested that the Wnt/ β -Catenin pathway regulates osteoclast differentiation by inducing the production of Osteoprotegerin (OPG), an inhibitor of RANKL that stimulates osteoclast formation (Yasuda, Shima et al. 1998). DKK1 has been shown to inhibit osteoblast differentiation and thus, decreases OPG

production by osteoblasts. As a result, RANKL is not inhibited and osteoclast formation occurs. The net result favors bone resorption at affected sites (Goldring and Goldring 2007). Other roles for DKK-1 have been described including cell-type specific regulation of cell migration (Koch, Capaldo et al. 2009; Kuang, Miao et al. 2009). These studies have shown that DKK1 acts to disrupt polarized cell migration by inhibiting the formation of cell polarity complexes necessary for directed movement in intestinal epithelial cells.

1.5. c-Jun N-Terminal Kinase-mediated Migration

JNK proteins are members of the MAP Kinase family of intracellular signaling molecules. To date, three genes have been described to code for the- three JNK proteins: JNK1, JNK2 and JNK3. JNK1 and JNK2 are both expressed ubiquitously while JNK3 is limited mainly to the brain (Gupta, Barrett et al. 1996). JNK proteins are serine/threonine kinases responsible for the transduction of signals through various intracellular pathways. JNK signaling contributes to numerous cellular functions including cell survival, scaffold arrangement, apoptosis, tumor development and migration (Pulverer, Kyriakis et al. 1991; Smeal, Binetruy et al. 1991; Xia, Dickens et al. 1995; Johnson, Spiegelman et al. 1996; Liu, Hsu et al. 1996; Adler, Yin et al. 1999; Behrens, Jochum et al. 2000; Cheng, Yang et al. 2000; Kurokawa, Mitani et al. 2000; Tournier, Hess et al. 2000).

Cell migration depends on numerous, simultaneous cellular processes including the assembly and disassembly of focal adhesions, as well as, directional polymerization and depolymerization of intracellular structural proteins (Cox and Huttenlocher 1998; Webb, Parsons et al. 2002). Recent studies have found that the specific phosphorylation

of paxillin by JNK at serine 178 is required for cell migration and the maintenance of adhesion turnover (Huang, Rajfur et al. 2003). A proposed mechanism by which JNK becomes activated depends on extracellular signals that activate Mitogen-activated protein kinase kinase kinase 1 (MEKK1). MEKK1 then phosphorylates the MAP kinase kinases, MKK4 and MKK7. These proteins are responsible for direct phosphorylation of JNK and subsequent activation of downstream effector molecules [53]. Significant data have been presented affiliating JNK with the migratory process. The use of a chemical JNK inhibitor, SP600125, inhibits cellular migration in numerous cell types (Huang, Rajfur et al. 2003; Javelaud, Laboureau et al. 2003; Kavurma and Khachigian 2003). The specificity of this drug and role of JNK in migration is confirmed in other experiments by showing a decreased rate of migration of JNK knockout cells as compared to wild-type (Pedram, Razandi et al. 2001; Zhan, Kim et al. 2003). A potential mechanism by which JNK promotes migration is dependent of the phosphorylation of paxillin and its subsequent polyubiquitination. Ubiquitination of paxillin could result in proteosomal degradation and a decrease in the cytoplasmic concentration of paxillin. As a result, the cell would show increased turnover of adhesions and a faster rate of migration.

1.6. The K/BxN Mouse Model of Rheumatoid Arthritis

In 1996, a novel mouse model of spontaneous arthritis was discovered by the group of Drs. Benoist and Mathis (Kouskoff, Korganow et al. 1996). Mice transgenic for a T cell receptor specific for bovine pancreatic RNase were bred onto a Non-obese diabetic (NOD) mouse and the offspring (K/BxN) spontaneously developed disease similar to that of rheumatoid arthritis. Further research identified that the development of

disease was B and T cell dependent and resulted in the production of autoantibodies towards glucose-6-phosphate isomerase (G6PI). T cells in this model show dual specificity towards both the bovine RNase epitope and G6PI. Although adaptive immune response is crucial for autoantibody development in this model, it has been shown that the innate immune response, namely Fc receptors, neutrophils, mast cells and other novel participants are required for disease development and severity (Corr and Crain 2002; Lee, Friend et al. 2002; Cramer, Yamanishi et al. 2003).

K/BxN mice express both the transgenic T cell receptor KRN as well as MHC class II IA^{g7} (Matsumoto et al. 1999). These mice produce autoantibodies reactive towards G6PI that accumulate in the distal joints (Korganow et al. 1999; Maccioni et al. 2002). The disease pathogenesis simulates some components seen in human rheumatoid arthritis and serves as a nice model for study of the autoimmune joint disease. A distinct advantage of this model is that disease is serum-transferrable from affected mice to recipient mice and serves as a means to study the effect of certain pathways using specific genetically deficient strains.

The working model of pathogenesis in this model includes participation of both adaptive and innate immune cells, with the effector phase dictated by innate immune responses. Initially, T-cells break tolerance to G6PI through binding to G6PI presented by the IA^{g7} MHC class II molecule on B cells. This results in the production of G6PI autoantibodies and the conversion of the B-cells to plasma cells. The autoantibodies react with G6PI in the distal joints and initiate an array of reactions resulting in neutrophil accumulation, mast cell activation and expansion of macrophages and fibroblast-like synoviocytes in the affected joint. These innate immune cells then produce inflammatory

cytokines, importantly IL-1 β and TNF α , that are thought to sustain the immune response and facilitate local joint destruction (Maccioni, Zeder-Lutz et al. 2002).

1.7. Hypothesis

Wnt gene expression profiles in RA tissues are elevated compared to normal tissues and could contribute to the unusual phenotype exhibited by the synovial lining during disease pathogenesis. Serum DKK1 levels are increased in rheumatoid arthritis patients and to date, has been primarily attributed to the aberrant bone-remodeling phenotype in RA patients. Here we examine the potential roles of DKK1 in pannus formation, the rapid proliferation and invasion of synovial cells into the joint tissues. We believe that the presence of Wnt signaling molecules, namely DKK1 and Wnt5a, characteristic of disease contribute to FLS migration into the joint tissues.

2. Methods

2.1. Reagents

Purified recombinant mouse Dickkopf-1, Wnt3a, and Wnt5a were purchased from R&D Systems (Minneapolis, MN). Calcein AM was purchased from Invitrogen (Eugene, OR). XGAL, 5-bromo-4-chloro-3-indoyl- β -galactopyranoside, was purchased from Genesee Scientific (San Diego, CA). EZ Run Pre-Stained Recombinant Protein Ladder was purchased from Fisher Scientific (Pittsburgh, PA). Phosphorylated JNK, total JNK, Non-phosphorylated β -Catenin, Phosphorylated β -Catenin and β -Actin antibodies were purchased from Cell Signaling Technology (Danvers, MA).

2.2. Mice

KRN T cell receptor (TCR) transgenic mice were a kind gift from Drs. D. Mathis and C. Benoist (Harvard Medical School, Boston, MA) and Institut de Génétique et de Biologie Moléculaire et Cellulaire (Strasbourg, France), and were maintained on a C57Bl/6 background (K/B). Arthritic mice were obtained by crossing K/B with NOD/Lt (N) animals (K/BxN). Progeny bearing the V β 6 transgenic TCR were identified by cytofluorometry of peripheral blood lymphocytes using anti-CD4 PE (Caltag, Burlingame, CA) and anti-V β 6 FITC (BD-PharMingen, San Diego, CA) labeled antibodies. C57Bl/6 mice were purchased from The Jackson Laboratories. TOPGAL β -Catenin- β -Galactosidase reporter transgenic mice were purchased from The Jackson Laboratories (Bar Harbor, ME). β -Galactosidase expression is regulated by three

LEF/TCF binding sequences upstream of a *c-fos* promoter. Expression is increased in the presence of LEF1/TCF3 signaling when coligated to activated β -Catenin characteristic of Wnt/ β -Catenin pathway activation. Mice were bred and maintained under standard conditions in the University of California, San Diego Animal Facility that is accredited by the American Association for Accreditation of Laboratory Animal Care. Tissues from *jnk1*^{-/-} and *jnk2*^{-/-} mice were a gift from Dr. Michael Karin (UCSD).

2.3. Serum Transfer and Arthritis Scoring

Arthritic adult K/BxN mice were bled and the sera were pooled. Recipient mice were injected with 150 μ l intraperitoneally (i.p.) as indicated in the figure legends on day 0. Clinical arthritis scores were evaluated using three methods. To quantify severity of arthritis a scale of 0–4 for each paw was implemented (0, normal; 1, minimal erythema and mild swelling; 2, moderate erythema and mild swelling; 3, marked erythema and severe swelling, digits not yet involved; 4, maximal and swelling, digits involved). Ankle thickness was measured with a caliper (Monostat, Switzerland) in mm and compared to ankle thickness prior to serum injection.

2.4. RNA Isolation and Quantitative PCR

RNA was isolated using Perfect Pure Fibrous Tissue RNA Kit (5 Prime, Gaithersburg, MD) following the manufacturer's instructions. After isolation, RNA stored at -80°C. 250ng of RNA was then used for synthesis of cDNA using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). Quantitative real-time PCR was performed using a BioRad iCycler (BioRad, Hercules, CA) using Taqman qPCR

gene expression assays (Applied Biosystems, Carlsbad, CA). Actin expression was used as a reference in all the qPCR experiments. Primers for: Wnt3a, Wnt5a, DKK1, β -Catenin, MMP3, VEGFa and Actin were designed and synthesized by Applied Biosystems (Carlsbad, CA).

2.5. Preparation of FLS

Adult mice were sacrificed and knees and ankles harvested. Joints were cut with a blade into small pieces and placed into a 50mL tube (BD Falcon, San Jose, CA). 15mL of Collagenase Media, 0.5mg/mL Collagenase Type VIII (Sigma Aldrich, St. Louis, MO) in RPMI-1640 (Sigma Aldrich, St. Louis, MO) was placed into the tube and incubated at 37°C with occasional mixing for two hours. Cells were washed and transferred to a T25 Flask (BD Falcon, San Jose, CA) and add 5mL of 10% FCS DMEM. The flasks were incubated at 37°C, 5% CO₂ until cells were confluent.

2.6. β -Galactosidase Chlorophenol Red Activity Assay

Adult TOPGAL transgenic and C57Bl/6 mice were selected based on age and sex similarity. Recipient mice were injected intraperitoneally (i.p.) with 150uL K/BxN serum. TOPGAL and C57Bl/6 non-recipient mice were used as negative controls for the experiment. Recipient mice were sacrificed on day 5, the average day of maximum inflammation, and the wrists and ankles harvested. The joints were snap frozen and pulverized. Pulverized tissue was resuspended in 100uL of PhosphoSafe® buffer (Merck KGaA, Darmstadt, Germany) and incubated on ice for 30 minutes. Samples were frozen until use at -20°C. Serial dilutions of β -Galactosidase were prepared in PhosphoSafe®

buffer and used as an activity standard with a high concentration value of 1 μ g/mL. In a 96 well plate, 100 μ L of cell lysate was added to 100 μ L of freshly made β -Galactosidase Substrate Solution (1mg/mL chlorophenol red galactopyranoside (CalBiochem/Merck KGaA, Darmstadt, Germany), 60mM sodium dibasic phosphate (Sigma Aldrich, St. Louis, MO), 10mM KCl (Fisher Scientific, Fair Lawn, NJ), 50mM β -mercaptoethanol (Sigma Aldrich, St. Louis, MO). Absorbance was read at 570nm in a multiwell plate reader (Molecular Dynamics, Sunnyvale, CA).

2.7. Cell Migration via Oris Cell Migration Assay Kit (Platypus Technologies, Madison, WI)

Early passage C57Bl/6 mouse FLS were cultured at 37°C in 10% fetal calf serum DMEM. Stoppers, included in the assay kit, were inserted into a 96 well plate to cover the central area of the wells. 100 μ L containing 12,000 FLS cells in 10% FCS DMEM were loaded into each well and allowed to adhere to the well overnight at 37°C. On day 1, the stoppers are pulled and media aspirated. 100 μ L of FCS DMEM with stimulatory agent was added and cells were allowed to migrate for 24hr. On day 2, the media was aspirated and replaced with 5mM Calcein AM in Delbucco's Modified Eagle Medium. Cells were incubated for 30 minutes at 37°C. Excess calcein was washed three times with DMEM. The fluorescent emission of cells located in the center of the plate was measured using a fluorescent plate reader set with an excitation wavelength of 495nm and emission wavelength of 515nm.

2.8. Wound Healing Assay

Early passage C57Bl/6 or JNK deficient mouse FLS were cultured at 37°C in 10% fetal calf serum in Delbucco's Modified Eagle Medium. Plates were marked on the bottom of the wells to establish consistent lateral bounds. 75,000 cells per well were plated in a 24 well flat bottom culture plate (Corning Inc., Corning, NY), or 15,000 cells per well on a 96 well flat bottom culture plate (Corning Inc., Corning, NY) in 10% FCS DMEM. The cells were allowed to settle for one day. On day 1, the media was aspirated and wells were washed with 500uL of 1X PBS. The cells were scratched perpendicular (one time in the 96 well plate, three times in the 24 well plate) to the marks on the bottom of the well and PBS aspirated. 200uL of media was added to each well containing either 2% or 10% FCS DMEM and a known concentration of purified protein. Following stimulation, the cells were photographed at specific locations localized to the boundaries on the bottom of the plate. These photos served as the initial wound size. After 24 hours, the cells were again visualized at the same sites and pictures were taken. The degree of cell migration was quantified by ImageJ software.

2.9. Quantification of Cell Migration Using ImageJ Software

Free ImageJ software was downloaded at the NIH website (<http://rsbweb.nih.gov/ij/>). Day 0 and Day 1 images of cell migration were uploaded into the program. Day 0 scratch areas were quantified following the cell edge and bounded by the lines drawn with permanent marker on the bottom of the well prior to the experiment. Day 1 wound areas were quantified following the cell edge and bounded by the same

lines corresponding to those of day 0. Areas were measured in pixels and used universally to compare separate experiments. To calculate relative migration, the wound area on day 1 was divided by the wound area on day 0. This gives a value corresponding to the fraction of wound area that had not been healed. This value was then subtracted from 1, corresponding to the area that recovered as a result of migration. The migration of C57Bl/6 FLS are used as a baseline for normal migration and all other fold-values of migration are determined by comparison to these values (i.e. wells stimulated with 50ng/mL of Wnt5a in 2% FCS DMEM are compared to wells treated with only 2% FCS DMEM).

2.10. Statistics

ANOVA with Bonferoni post-test analysis for comparison of means was performed with Prism 5 for Macintosh (GraphPad Software, San Diego, California).

3. Results

3.1. Wnt pathway proteins are affected in a timecourse of serum transfer arthritis

K/BxN serum injection causes spontaneous arthritis disease development in the distal joints of treated mice. As a means to study the pathogenesis of arthritic disease, we implemented this mouse model to gain insight into the potential protein contributors to disease. In this experiment, C57Bl/6 mice were injected with 150uL of K/BxN serum and the severity of inflammation was measured using ankle thickness and arthritis score measurements. Figure 3 (A) shows the average change in ankle thickness for each mouse as compared to their initial day 0 measurement. After K/BxN injection, ankle thickness steadily increased through day 3 where it was maintained to day 5 and nearly resolved by day 10. The clinical arthritis score for paw swelling demonstrated the same trend.

To examine the expression of Wnt pathway associated proteins the expression of selected mRNA amplicons was assessed at different timepoints over the course of serum transferred arthritis. Groups of mice were injected with K/BxN serum and sacrificed at different timepoints. Figure 3 (B) shows quantitative PCR analysis of mRNA isolated from K/BxN serum injected paws over the timecourse. Wnt ligands, Wnt3a and Wnt5a, are upregulated 8-fold and nearly 3-fold, respectively. Cumulative joint β -Catenin transcriptional activity steadily dropped over the timecourse. Matrixmetalloprotease 3 showed the most marked increase with nearly 40-fold induction by day 5. DKK1 is also

actively transcribed showing no initial change in transcriptional activity followed by a 4-fold increase by day 5. Vascular endothelial growth factor a showed a decrease initially followed by a steady increase in transcription over the timecourse.

3.2. β -Catenin inducible protein expression increases over the timecourse of serum transfer arthritis

Synovial cells from arthritis patients show unusually high proliferation and invasive behavior (Gay et al. 1993). There is also evidence to suggest that the Wnt pathways were involved in pathogenesis of disease (Sen et al. 2000). To test if the Wnt/ β -Catenin pathway is involved was K/BxN serum mediated arthritis we injected 150uL of K/BxN serum i.p. into both C57Bl/6 and TOPGAL transgenic mice which expressed β -Galactosidase under control of the LEF/TCF binding domain, which is activated by β -Catenin/TCF binding. On days 0, 1, 2, 3 and 4, mice were sacrificed and paws were harvested and snap frozen in liquid nitrogen. Paws were then pulverized, cells lysed and proteins collected. The protein samples were analyzed for β -Galactosidase activity using a chlorophenol red activity assay. Figure 4 shows that although TOPGAL cells showed a basal level of β -Galactosidase expression, injection with K/BxN serum significantly increased LEF/TCF inducible protein expression. C57Bl/6 mice were used as a negative control to validate that activity was due to β -Galactosidase expression as opposed to background activity of the substrate solution.

3.3. C57Bl/6 fibroblast-like synoviocytes migrate *in vitro* in response to wounding

Synoviocytes in rheumatoid arthritis patients show characteristic proliferation, also known as pannus formation. Using cultured FLS, we needed to first show that these cells are capable of variable migration *in vitro* in order to elucidate the potential roles that Wnt proteins may play in the initiation of migration. As a preliminary experiment, we wished to assess the role of nutrient deprivation on cell movement. Here cultured cells were subjected to wounding and migration into the wound area was assessed.

Figure 5 shows that FLS given 10% FCS in DMEM were able to migrate more readily than those given lower concentration fetal calf serum. The absence of FCS resulted in little to no migration. The change in fluorescence value accounts for the difference in fluorescence within the wound area between day 0 and day 1 of the experiment. Treatment with 2% FCS resulted in reliable migration that was slightly less pronounced than when greater concentrations of FCS were offered. Based on this knowledge, we hypothesized that proteins that promote migration could be more easily discerned from those that did not when the stimulation was done in low, but not depleted, FCS concentrations (i.e. 1-2%).

3.4. Dickkopf-1 induces migration of C57Bl/6 FLS *in vitro*

The purpose of this experiment was to explore potential Wnt proteins and antagonists that may contribute to the induction of synoviocyte migration. Confluent cultures of FLS were wounded by scratching and subsequently treated with wnt ligands,

Wnt3a, Wnt5a or the canonical Wnt pathway antagonist Dickkopf-1 at a concentration of 30ng/mL in 1% FCS DMEM and incubated for one day at 37°C.

Figure 6 (A) shows an analysis of the 'Change in Fluorescence' between the treated and untreated synoviocytes, suggests that Wnt5a (30ng/mL) and, surprisingly, DKK1 (30ng/mL) initiated migration in the cells *in vitro*. Without treatment, cells migrated to a change in fluorescence value of about 100 units. Treatment with the Canonical Wnt ligand Wnt3a (30ng/mL) resulted in similar fluorescence values of just over 100 units suggesting that the activation of the Wnt/ β -Catenin pathway did not stimulate migration in FLS. Upon analysis of Wnt5a-treated positive control cells, it was confirmed that Wnt5a induces migration in FLS. These cells show nearly double the movement into the wound area when compared to the untreated negative controls. Most notably, DKK1 treatment induced migration in synoviocytes *in vitro*. Similar to the Wnt5a-treated cells, DKK1 induced migration over twice as much as the untreated negative control and shows statistical significance when compared to the untreated cells. Figure 6 (B) shows images of untreated, Wnt5a and DKK1 treated cells in 2% FCS DMEM. Wnt5a and DKK1 migrated more readily than untreated cells.

3.5. Wound edge FLS show JNK1 and JNK2 activation *in vitro*

As previously mentioned, JNK protein has been shown to act as a mediator of cell migration. Our hypothesis was that JNK is activated in response to wounding and that

this activation may contribute to the initiation of migration in wound edge synoviocytes. Figure 7 shows western blot analysis of cultured FLS before and after wounding. Confluent wells of cells were scratched horizontally and vertically leaving multiple wound-edges. The cells were lysed, the proteins separated on a gradient gel and then transferred to a membrane.

JNK1 phosphorylation occurs rapidly after wounding and is sustained for at least two hours. The most prominent JNK1 activation occurs early after 5 minutes suggesting that it is the initial isoform affected following wounding. JNK2 is also activated after wounding showing a prominent band after 30 minutes. JNK2 activation is sustained until at least 2 hours following wounding and returns to basal levels overnight. Untreated cells generally require 2 days to completely migrate into the wound area. These data suggest that both JNK1 and JNK2 are activated in response to wounding and may be important in the initiation but not maintenance of cell migration into the cell void area.

Phosphorylated β -Catenin levels were also followed over the timecourse using an antibody targeting phosphorylation at threonine 41 or serine 45. Serine 45 is phosphorylated by Casein Kinase I as a priming event for subsequent phosphorylation by GSK3 β at threonine 41 and degradation at the proteasome. Following wounding, β -Catenin is rapidly destabilized by phosphorylation at these residues indicating that canonical Wnt/ β -Catenin signaling is inhibited after wounding.

3.6. JNK1 and JNK2 deficient FLS show attenuated migration

Based on the information that JNK1 and JNK2 are both activated in response to wounding, we decided to analyze the role of these two JNK isoforms individually on the migration of FLS. In this experiment, confluent cells were scratched and the degree of wound closure was measured over a two day period. Figure 8 shows that on day 2, C57Bl/6 FLS showed an average wound closure of nearly 60% of the wound area. JNK1 deficient FLS showed a statistically significant attenuation of migration showing just over 20% closure of the wound area. JNK2 deficient FLS showed a similar, statistically significant attenuation of migration with wound closure at just over 20% of the area. The data suggested that both JNK1 and JNK2 are important for synoviocyte migration *in vitro* and that one does not easily compensate for the absence of the other isoform.

3.7. DKK1 stimulation restores migration in JNK1, but not JNK2 deficient FLS

As shown previously, DKK1 stimulation induces migration in C57Bl/6 fibroblast-like synoviocytes *in vitro*. Analysis of a wound assay of cultured synoviocytes by western blot identified JNK1 and JNK2 activation in response to wounding. Subsequent migration analysis of JNK1 and JNK2 knockout mice showed that the absence of either isoform attenuated the ability of synoviocytes to migrate *in vitro*. The purpose of this experiment was to determine whether DKK1 stimulation could restore migratory behavior in JNK knockout FLS.

Figure 9 shows the response of JNK1 and JNK2 cells to DKK1 stimulation after one day *in vitro*. On day 1, C57Bl/6 wild type synoviocytes migrate and close about 40% of the wound area. JNK1 and JNK2 show a pronounced decrease in migration, closing only about 25% and less than 20% of the wound area on day 1, respectively. After treatment of the cells with DKK1 at a concentration of 50ng/mL, the C57Bl/6 and JNK1 deficient synoviocytes migrated to around 60% of wound closure after 1 day. The DKK1-induced migration of JNK1 deficient FLS proved to be statistically significant when compared to the untreated JNK1 deficient FLS. JNK2 deficient cells did not respond to treatment with 50ng/mL DKK1 on day 1 and showed no statistical difference to the untreated JNK2 knockout FLS. This suggests that DKK1 likely induces FLS migration via a mechanism dependent on JNK2.

3.8. Immunoblot analysis of C57Bl/6 FLS stimulated with DKK1 or Wnt5a

To verify that Wnt pathway induced migration by DKK1 involved a mechanism through JNK2 activation, an immunoblot analysis was conducted on FLS stimulated with 50ng/mL of DKK1 over a 2 hour timecourse.

Figure 10 (Upper Panel) shows the absence of other stimuli, DKK1 induced JNK2 after 5 minutes and maintained activation over the 2 hour timecourse when compared to the unstimulated 0 minute timepoint. Figure 10 (Lower Panel) includes the positive control for the experiment, a separate immunoblot was completed with Wnt5a stimulation

at 50ng/mL. As expected and previously demonstrated, JNK proteins were phosphorylated and activation was sustained through the 2 hour timecourse.

4. Discussion

The data supplied in this thesis demonstrates an additional role that Wnt signaling might in the pathogenesis of rheumatoid arthritis. It is known that human RA synovial tissues express elevated levels of Wnt-associated transcripts as compared to normal tissue or osteoarthritis synovia, suggesting that Wnt signaling may have a distinct role in human disease (Sen et al., 2000). Other labs have also shown that Wnt/Frizzled signaling through the canonical pathway could contribute to hyperproliferative diseases through constitutive activation (Logan and Nusse, 2004). This is especially important when considering the characteristic hyperproliferative phenotype exhibited by synoviocytes in RA tissues. K/BxN serum injection resulted in similar expression profiles of Wnt-associated genes to human disease and may be a useful tool to study Wnt pathway contribution to disease. Upregulation of Wnt3a, canonical Wnt pathway activator, and Wnt5a were marked and peaks correlated with disease severity. Notably, Dickkopf-1 expression also increased following serum transfer making it another potential contributor to disease.

Of note in this thesis is the introduction of a new role for the secreted protein Dickkopf-1 in the induction of migration of FLS *in vitro*. Treatment of FLS with Dickkopf-1 in a wound healing assay resulted in a statistically significant increase in the migration of wound edge synoviocytes. Although cell migration is likely the result of numerous pathways working in conjunction with each other, analysis of the mechanism by which migration is induced suggests that c-jun N-terminal Kinase (JNK) is important in the initiation of synoviocyte migration. Western blot analysis of wound edge,

migrating FLS *in vitro* shows marked JNK isoform activation. In addition, the absence of either JNK1 or JNK2 attenuates migration of FLS *in vitro*.

Interestingly, treatment of JNK1 knockout synoviocytes with Dickkopf-1 was able to re-establish the migratory behavior of these cells. JNK2 knockout synoviocyte migration was not induced upon stimulation with Dickkopf-1. These results suggest that Dickkopf-1 likely signals through JNK2 to induce migration in wound edge synoviocytes and that the absence of JNK2 renders DKK1 ineffective with regards to migration. Recently, Dickkopf-1 has been described as having the ability to induce migration in HEK293 cells. The mechanism shown involved reducing cell-cell adhesions through β -Catenin and Cadherin degradation (Kuang et al., 2009). Here we provide evidence that migration of C57Bl/6 FLS *in vitro* is JNK2 dependent and may present another level of sophistication to the mechanism of action. To date, a direct connection linking JNK signaling to Cadherin complex stability has not been made suggesting that perhaps the two pathways both contribute to the migratory behavior of FLS in RA.

A notable aspect of these findings is that DKK1 could become a new therapeutic target for disease intervention. As discussed earlier, rheumatoid arthritis is characterized by hyperproliferation and invasion of synovial tissue into the joint matrix. Wnt signaling is elevated in RA tissues and could be contributing to the aberrant phenotype of pannus formation exhibited by synoviocytes. High Wnt3a expression levels could account for cell hyperproliferation while elevated DKK1 promotes their migration. This is supported by the fact that DKK1 is Wnt3a-inducible (Niida, Hiroko et al. 2004), and that both genes show elevated expression during disease progression in the K/BxN mouse model of disease. Reduced serum levels of DKK1 could result in decreased migratory activation of

FLS and hinder pannus formation during early disease. The return of DKK1 to basal serum levels could also limit bone resorption in RA affected joints (Diarra et al. 2007). DKK1 has been shown to inhibit osteoblast differentiation, indirectly elevating RANKL levels and promoting osteoclast formation (Goldring and Goldring 2007).

Although much still needs to be determined regarding the role of DKK1 in the pathogenesis of rheumatoid arthritis, the introduction of DKK1 as a promoter of FLS migration proposes a new mechanism by which FLS invasion could be initiated. Figure 11 shows possible mechanisms through which DKK1 induced FLS migration could occur. With this knowledge, DKK1 could become a new potential target for disease intervention by presenting a non-immunosuppressive approach to RA treatment specifically directed at correcting the aberrant behavior of RA synoviocytes.

Appendix

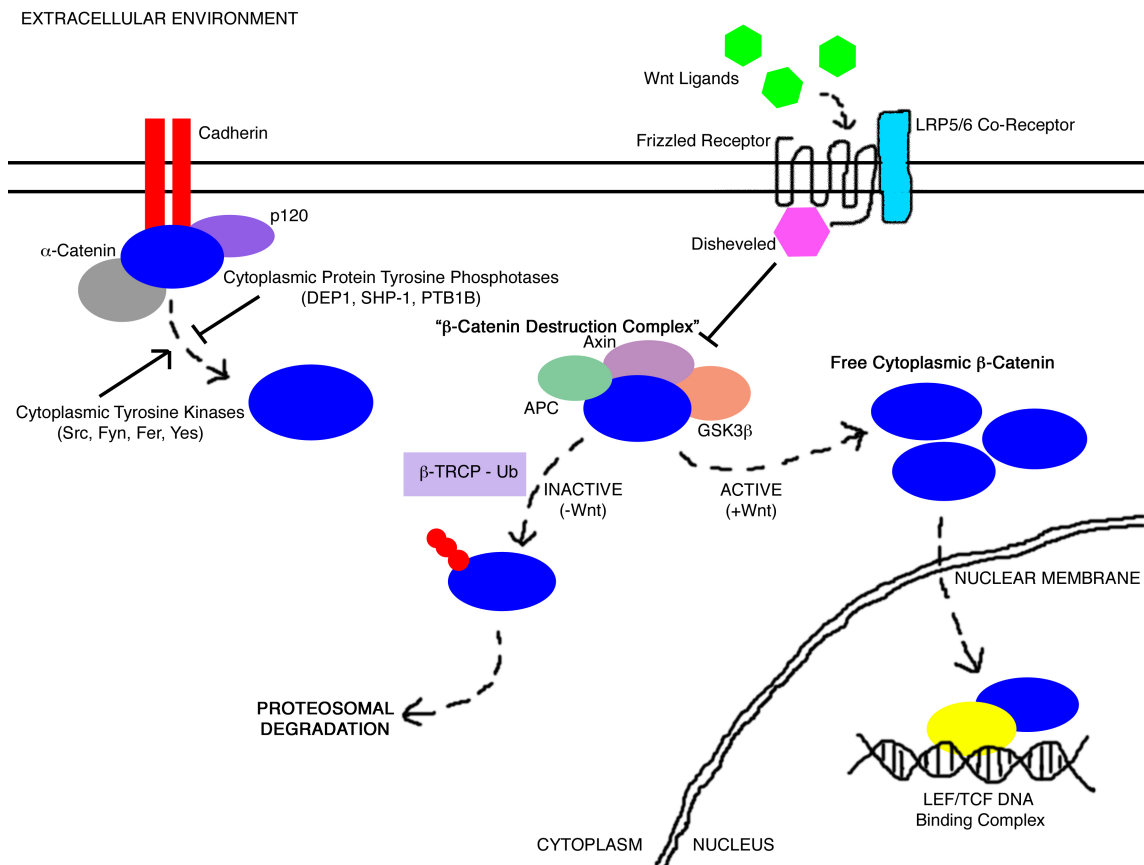


Figure 1: Wnt/β-Catenin Signaling.

β-Catenin resides in three major cellular compartments within the cell: the cell membrane, the cytoplasm and the nucleus. At the membrane, β-Catenin forms a complex with Cadherins, p120 and α-Catenin. The presence of this membrane complex positively regulates extracellular cell-cell adhesions. Complex formation is regulated by the activity of numerous cytoplasmic kinases and phosphatases. Kinase activity at tyrosine residues promotes dissociation of β-Catenin from the membrane complex while tyrosine phosphatase activity is necessary for the maintenance of the membrane bound complex of proteins. Cytoplasmic β-Catenin exists in two main states. Free cytoplasmic β-Catenin is not associated with any other protein complexes and capable of translocation into the nucleus. It may also be bound by the “β-Catenin Destruction Complex” composed of APC, Axin, GSK3β and β-Catenin. The presence of Wnt/β-Catenin activation inhibits complex formation and favors the accumulation of cytoplasmic β-Catenin. The absence of Wnt/β-Catenin signaling results in polyubiquitination of β-Catenin and subsequent degradation at the proteasome. The final compartment for β-Catenin is the nucleus. Free cytoplasmic β-Catenin translocates into the nucleus and activates transcription of β-Catenin inducible genes at LEF/TCF binding domains.

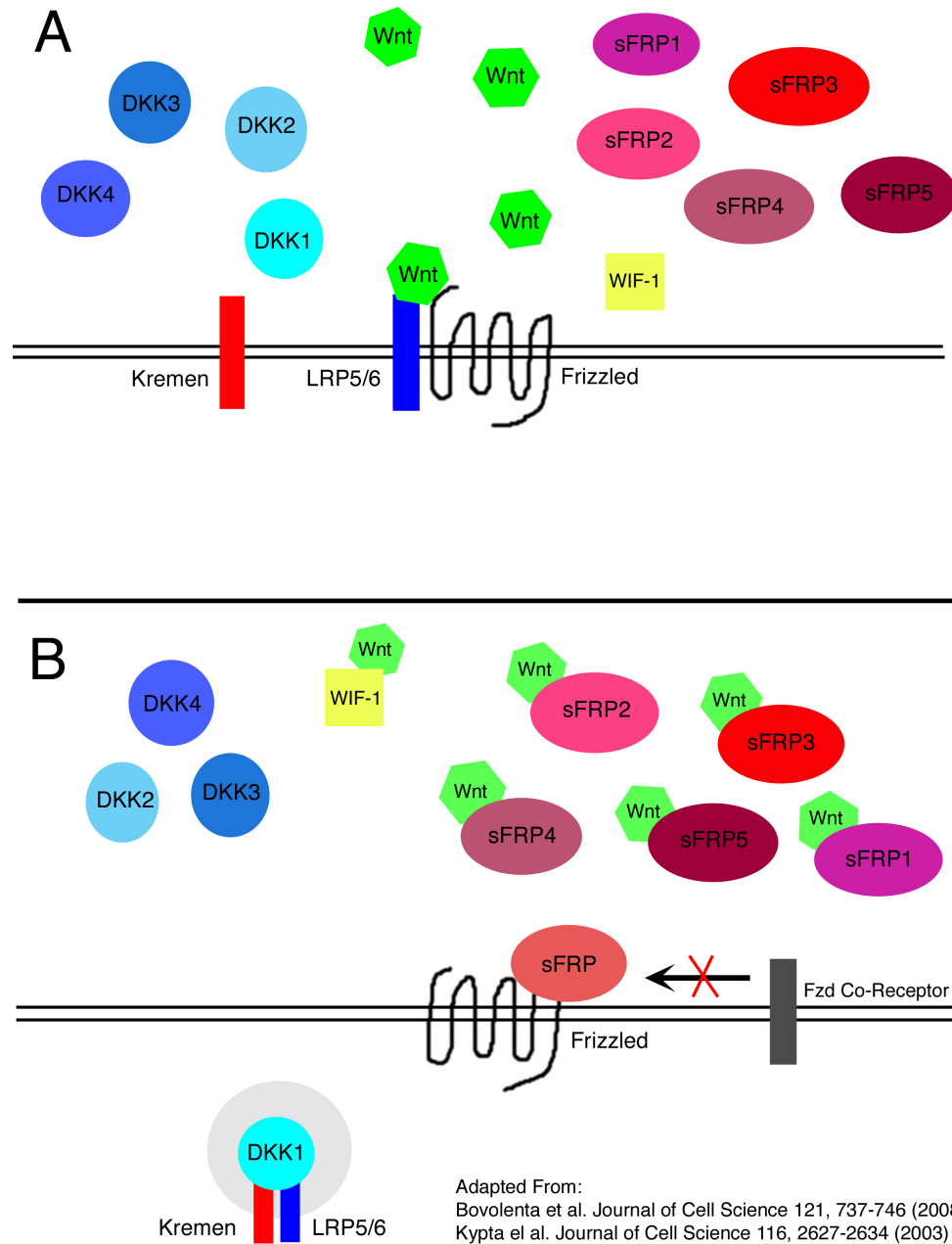


Figure 2: Extracellular Wnt Inhibitors.

Numerous extracellular Wnt protein inhibitors exist. Four Dickkopf family proteins exist and are described to inhibit canonical Wnt signaling by binding to the LRP5 frizzled co-receptor in conjunction with Kremen to initiate clathrin-dependent internalization of the receptor. Secreted Frizzled-related Proteins (sFRP's) contain a cysteine-rich domain (CRD) that shares high structural homology to the Frizzled receptor. These Wnt inhibitors act by binding to and competitively sequestering them from binding their membrane receptors. It has also been shown that sFRP is capable of inhibiting Wnt signaling by binding directly to the Frizzled receptor and inhibiting co-receptor association. Wnt Inhibitory Factor-1 (WIF-1) acts in a similar manner by binding to and blocking Wnt interaction with its membrane receptors.

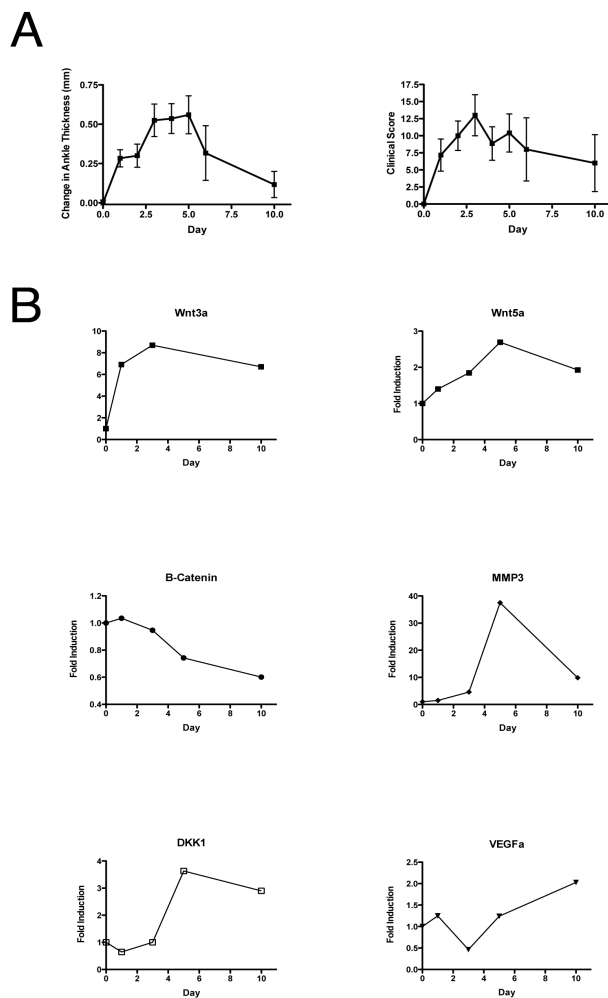


Figure 3: Wnt pathway gene expression signature during a timecourse of serum transfer arthritis.

C57Bl/6 mice were injected with 150 μ L of K/BxN serum intraperitoneally on day 0 and groups of 3-4 mice were sacrificed on days 0, 1, 3, 5 and 10 over the timecourse. (A) Each day, the change in ankle thickness for the surviving mice was measured with a caliper and arthritis was scored. The average change in ankle thickness for each animal as compared to the day 0 measurement. (B) Ankles from mice sacrificed on the indicated days were harvested, pooled and snap frozen at -80°C . Ankles were then pulverized and RNA was isolated. Quantitative PCR analysis was completed on Wnt3a, Wnt5a, β -Catenin, MMP3, DKK1, VEGFa and Actin. Shown are fold induction for each amplicon compared to the Day 0 expression levels, following normalization to actin levels.

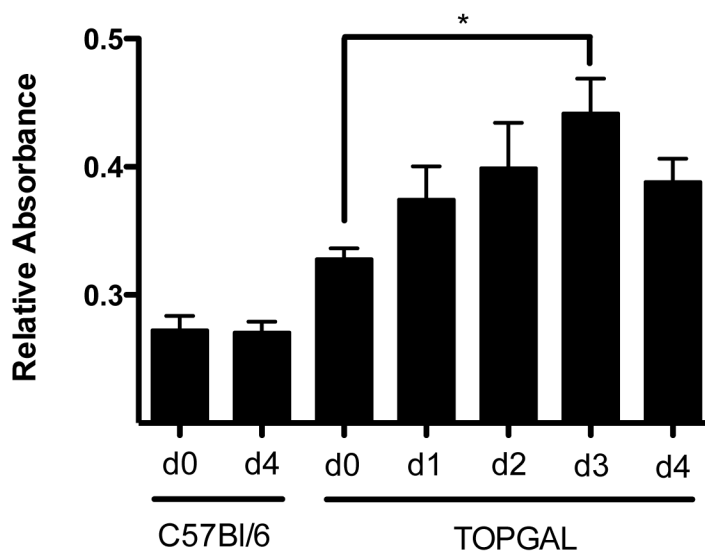


Figure 4: TCF/β-Galactosidase reporter gene expression during a timecourse of serum transfer arthritis.

C57Bl/6 and TOPGAL β-Galactosidase-transgenic mice were injected with 150uL of K/BxN serum intraperitoneally on day 0 and two mice per group were sacrificed on days 0, 1, 2, 3 and 4 over a 4 day timecourse. Ankles of mice harvested and snap frozen at -80°C. Ankles were pulverized and lysed in 100uL lysis Buffer for 10 minutes. Lysate was transferred to a 96 well plate and 100uL of β-Galactosidase Substrate Solution was added. Absorbance was read at 570nm after development. * denotes $p < .05$ by ANOVA and Bonferroni correction.

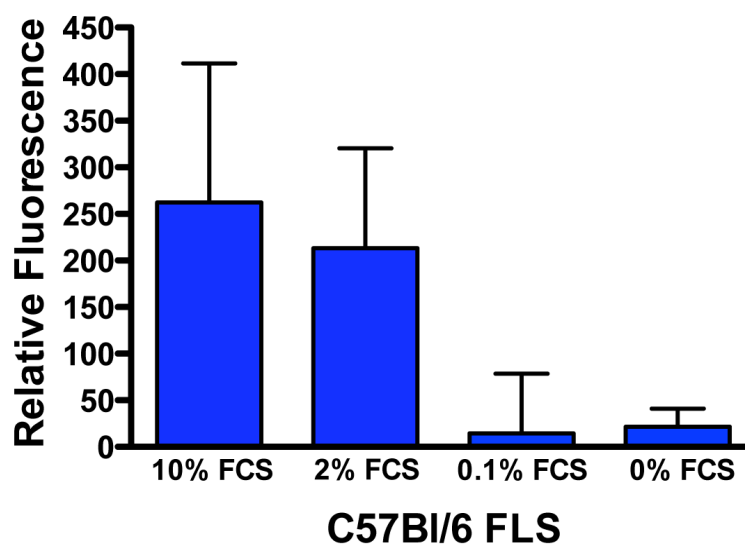


Figure 5: Migration is serum concentration dependent in C57BI/6 FLS.

Confluent planes of C57BI/6 FLS were plated in a 96 well plate with center-well blockers inserted and allowed to adhere overnight. The next day the blockers were pulled and the cell media was replaced with varying concentrations of fetal calf serum. The cells were allowed to migrate for 24 hours. The next day the cells were stained with calcein AM and fluorescent intensity in the center of the wells was measured at 495nm.

A

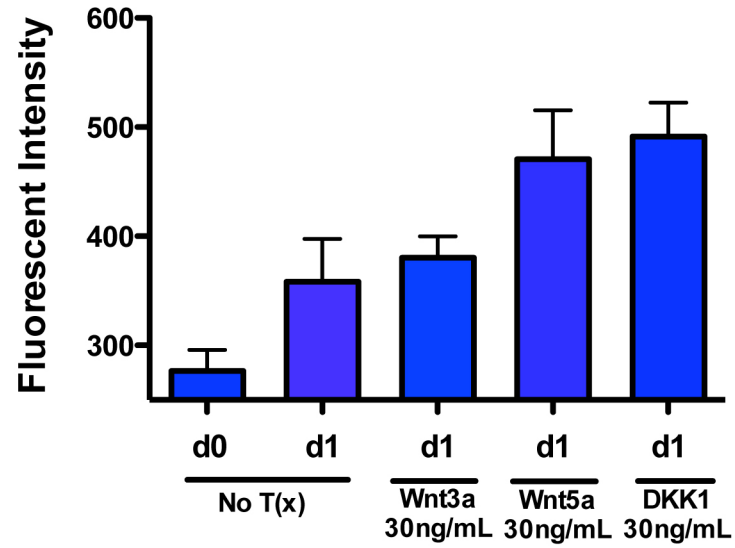


Figure 6: C57Bl/6 FLS migration after stimulation with Wnt pathway proteins.

(A) Confluent planes of FLS were wounded and subsequently treated with Wnt pathway associated molecules: Wnt3a, Wnt5a, or Dickkopf-1 at a concentration of 30ng/mL in 1% FCS DMEM and incubated for 1 day at 37°C. The next day, the cells were stained with Calcein AM and fluorescent intensity was measured at 495nm. $p < 0.001$ for the experiment with $n=3-6$ wells per group.

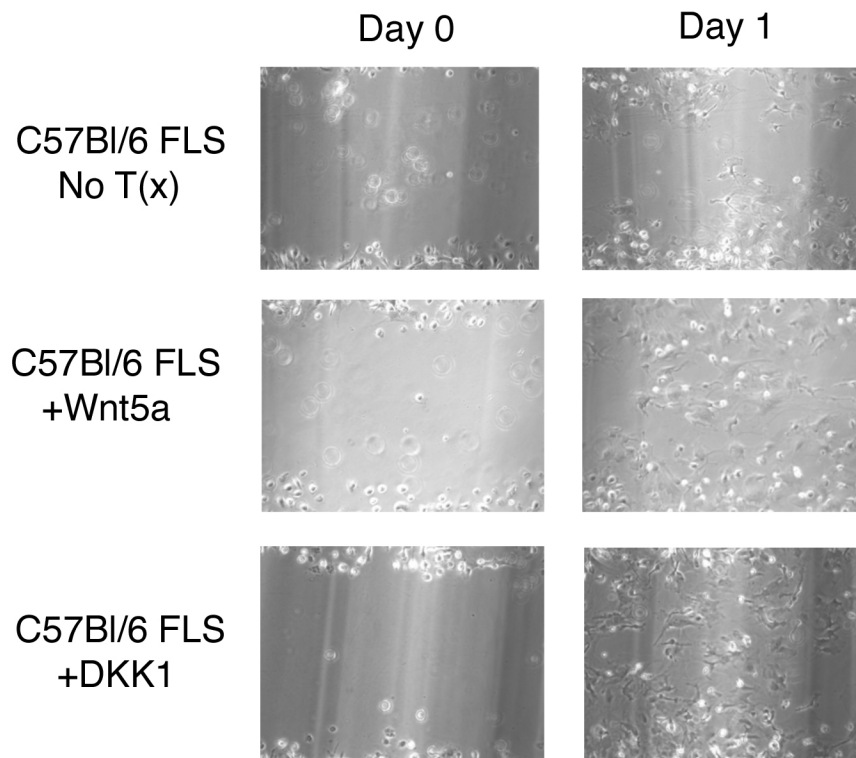
B

Figure 6: C57Bl/6 FLS migration after stimulation with Wnt pathway proteins.

(B) Migration pictures of a scratch assay of FLS without treatment, with 50ng/mL Wnt5a or with 50ng/mL of DKK1 in 2% FCS DMEM.

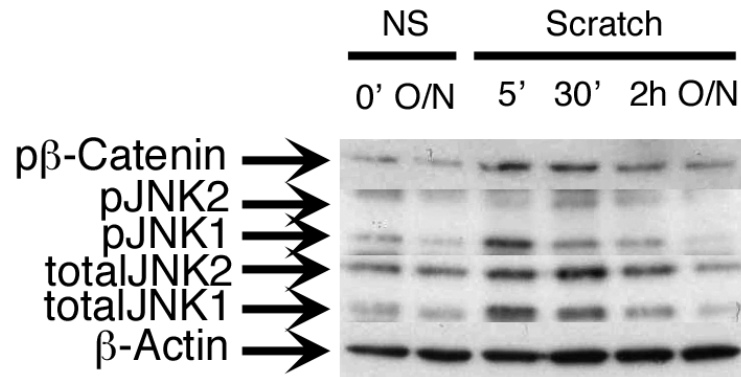


Figure 7: Immunoblot analysis of wound edge C57Bl/6 FLS *in vitro*.

Confluent planes of C57Bl/6 FLS were plated on a 24 well plate in 10% FCS DMEM. Cells were scratched three times each horizontally and vertically with a pipette tip and lysed at specific timepoints. Five ug of lysate from each timepoint was separated on a 12 well 4%-12% gradient gel and transferred to a membrane for immunoblot analysis. The membrane was probed with the indicated antibodies.

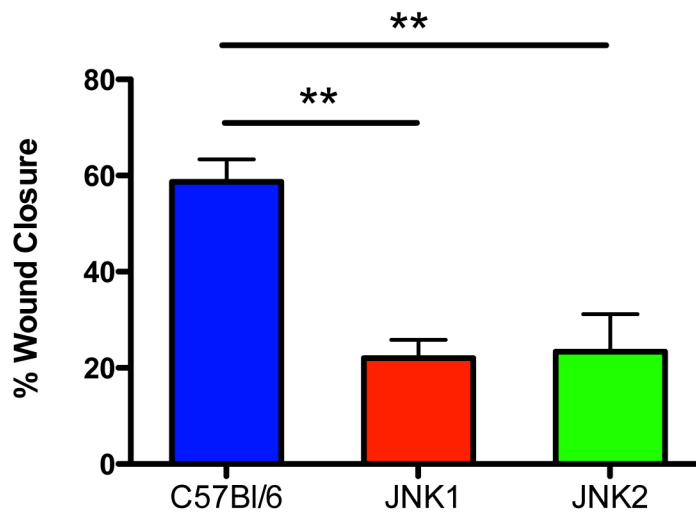


Figure 8: JNK1 and JNK2 deficient FLS have limited migration.

C57BI/6, JNK1 knockout and JNK2 knockout FLS were plated to confluency in a 96 well plate in 10% FCS DMEM and allowed to adhere overnight. Media was replaced with 2% FCS DMEM and the confluent plane was scratched. The wells were imaged at previously marked sites at baseline and after one day. % Wound closure was determined by quantification of the wound areas initially and after a single day using ImageJ software. $p < 0.002$ for the experiment with $n=3-6$ wells per group.

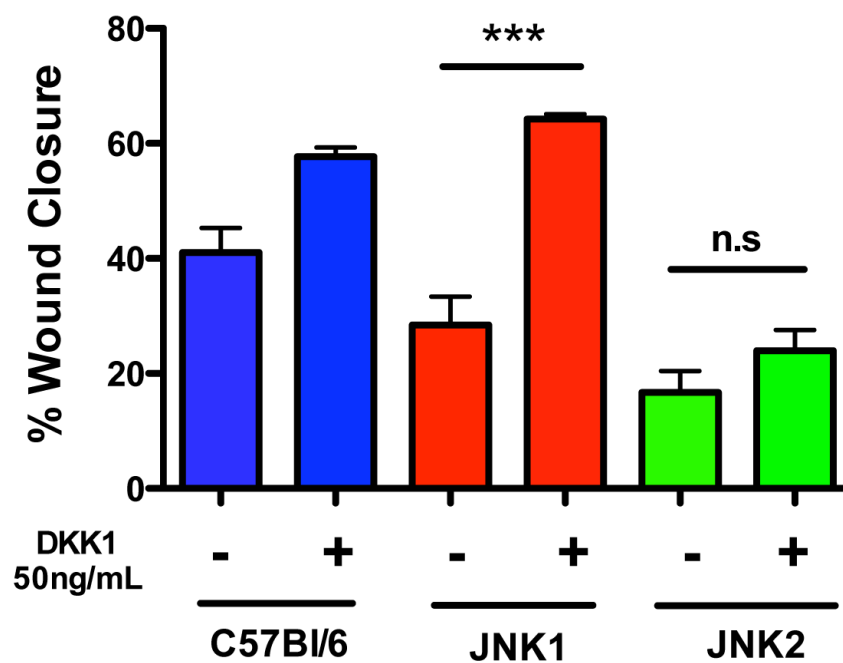


Figure 9: JNK1 and JNK2 deficient FLS migration with DKK1 stimulation.

C57Bl/6, JNK1 and JNK2 knockout FLS were plated to confluency in 10% FCS DMEM in a 96 well plate and allowed to adhere overnight. The next day, the cells were wounded and the media was aspirated and replaced with either 2% FCS DMEM or 50ng/mL DKK1 in 2% FCS DMEM. Images were taken initially after wounding and again 1 day later. % Wound closure was quantified by comparing the wound area of migrating cells at day 1 to the initial area on day 0. *** denotes $p < 0.0001$ with $n = 4$ per group.

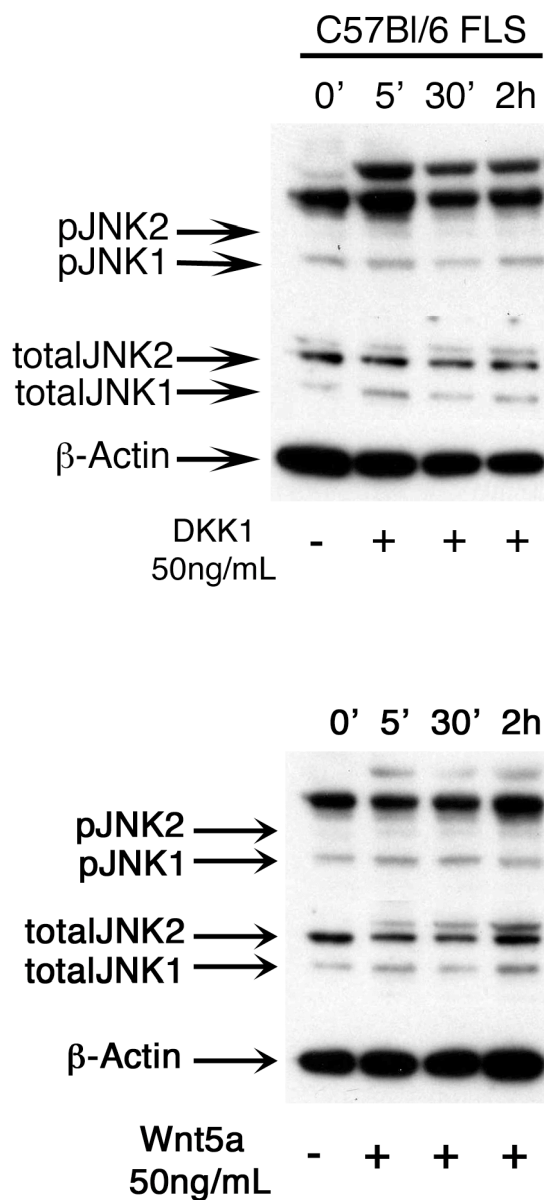


Figure 10: Immunoblot analysis of C57Bl/6 FLS stimulated with DKK1 or Wnt5a.

C57Bl/6 FLS were plated to confluency in 10% FCS DMEM in a 24 well plate and allowed to adhere overnight. The next day, the cells were stimulated with 50ng/mL of DKK1 or Wnt5a in 10% FCSDMEM and incubated at 37°C. Cells were lysed at specific timepoints 0', 5', 30' and 2h and lysates were separated by SDS-PAGE and then transferred to a blotting membrane. The membranes were serially probed with the indicated antibodies.

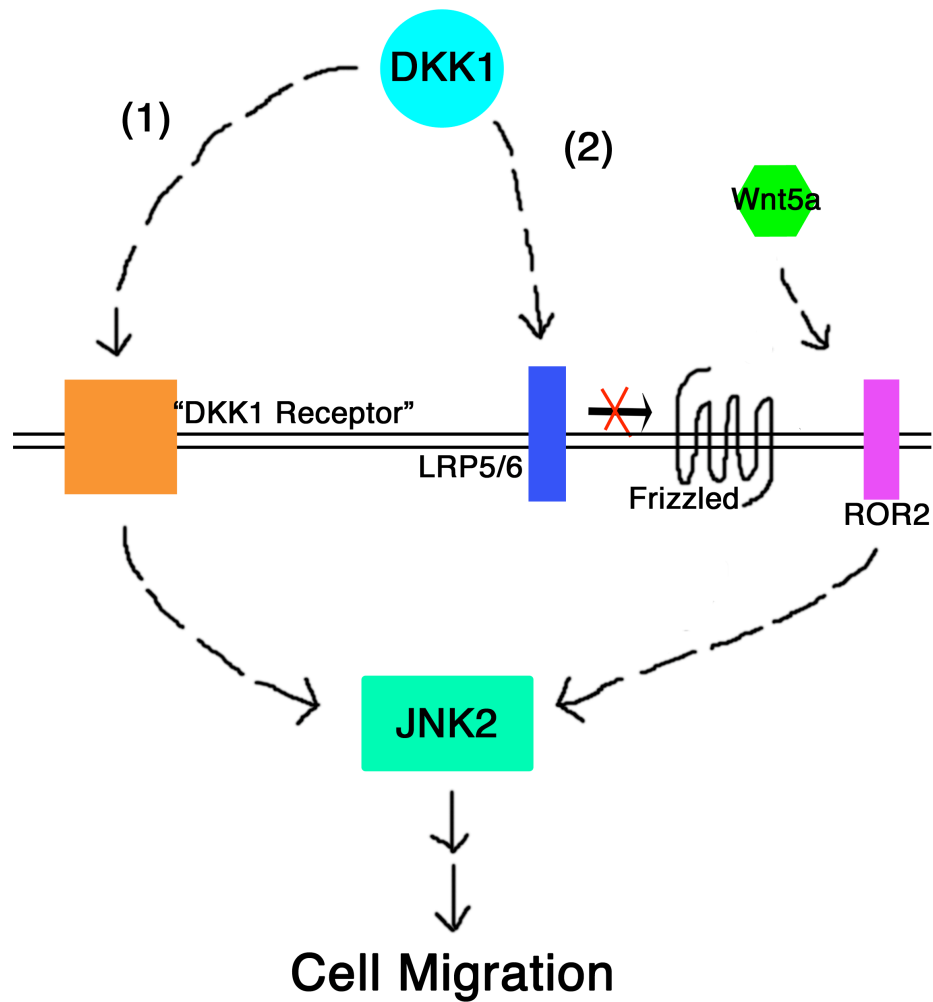


Figure 11: Hypothesis.

DKK1 stimulation of C57Bl/6 FLS induces migration *in vitro*. Although the exact mechanism is not known, the results of this thesis suggest an alternative role for DKK1 apart from Wnt/ β -Catenin inhibition. To date, DKK1 has only been described to bind cell surface receptors Kremen and LRP5/6 but, based on prior information published regarding DKK1 and canonical signaling, it is likely that DKK1 is working either: (1) independently of previously described pathways through a different cell surface receptor or (2) there exists a level of cross-talk between canonical and non-canonical pathways.

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