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Dickkopf1: A tumor suppressor or metastasis promoter?

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Abstract

Dickkopf1 (DKK1), a secreted inhibitor of the Wnt/β-catenin pathway, is a negative regulator of bone formation. DKK1 acts as a switch that transitions prostate cancer bone metastases from osteolytic to osteoblastic and also is an active indicator of poor outcome for multiple myeloma. However, in other tumor types, DKK1 up-regulation or over-expression suppresses tumor growth. Thus, the role of DKK1 in cancer appears to be diverse. This raises a question: Could the increased levels of DKK1 still be tumor protective when observed in high levels in the serum of patients? Here, we summarize the diverse, seemingly contradicting roles of DKK1 and attempt to explain the apparent dichotomy in its activity. We propose that DKK1 is a critical secreted factor that modulates microenvironment. Based on the location and components of the microenvironment DKK1 will support different outcomes.

Wnt factors induce several downstream signaling responses. Many of these responses are extensively studied, evolutionarily conserved and involve an intricate network of signaling molecules known to play an essential role in development [1-3]. Canonical Wnt pathway (Wnt/β-catenin pathway) follows a series of well described molecular events following binding of Wnt ligands to the Frizzled receptor and co-receptor low density lipoprotein receptor related protein (LRP5/6). This binding prompts Dishevelled (Dsh) mediated inhibition of the destruction complex for TCF/LEF transcription co-factor β-catenin and transcriptional activation of downstream target genes such as c-Myc and Cyclin D1. Precise regulation of this network is necessary for appropriate cellular function. Deregulation of the Wnt pathway has been implicated in several diseases including bone diseases, Alzheimer's disease and cancer [2, 4-7]. One of the ways Wnt signaling is precisely regulated in cells is by a delicate balance of extracellular agonists and antagonists. There are at least 7 known antagonist protein groups that modulate Wnt pathway function. The secreted Frizzled-Related Proteins (sFRPs) [8], Cerberus [9], Crescent (frzb2 frizzled-related protein 2) [10] and Wnt Inhibitory Factor-1 (WIF-1) [11] inhibit Wnt signaling by binding to and sequestering Wnt ligands, thus preventing Wnt ligands from binding to and activating their cognate cell surface receptors. Wise (Wnt modulator in surface ectoderm, known as Sclerostin Domain Containing 1, SOSTDC1) inhibits the Wnt pathway depending on the cellular context either by competing with Wnts for interaction with the Wnt co-receptor, LRP6 [12] or by reducing cell surface presentation of LRP6 by retaining it in endoplasmic reticulum, causing inhibition of Wnt signaling [13]. NDK1 (naked cuticle homolog 1) binds to Dsh and abolishes its function leading to inhibition of Wnt signaling [14-16]. The last group of Wnt antagonists, the Dickkopf family (DKK) of proteins, is structurally unrelated to Wnts or Frizzled. DKK family members are secreted Wnt inhibitors that bind to and sequester the Wnt co-receptors LRP5/6, to inhibit Wnt signaling [17-19]. The human DKK family of proteins consists of five evolutionarily conserved members, DKK1, DKK2, DKK3, DKK4 and a unique DKK3-

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related member, DKKL1 (Dickkopf-like protein 1, Soggy). In a comprehensive review Niehrs *et al*. have discussed DKK family members, their evolution, function and receptors [20]. In this review, we will limit our discussion to the first family member, Dickkopf homolog 1 (DKK1) and its role in tumor biology.

Dickkopf homolog 1 (DKK1) was first identified in *Xenopus* while screening for factors capable of inducing head formation [21] [Dickkopf: German for big head]. Fedi *et al*. discovered the human DKK1 homolog [22]. Human DKK1 is N-linked glycosylated on the carboxy terminal region [23]. Two different models have been proposed to account for the mechanism by which DKK1 antagonizes LRP6 function. One says that it binds to the Wnt co-receptor LRP5/6 and transmembrane proteins KREMEN1 and KREMEN2, forming a ternary complex that causes endocytosis and removal of LRP5/6 from the plasma membrane. As a result, LRP5/6 is unavailable for interaction with Wnt ligands and Frizzled and formation of the signaling receptor complex is hindered. Ultimately, this inhibits extracellular activation of Wnt signaling [17, 24-26]. Other model proposes that DKK1 binds to and sequesters LRP6, which disrupts Wnt-induced Frizzled-LRP6 complex formation while KREMEN may play only a marginal role [27-29]. Exhaustive studies by Semenov *et al* demonstrated that DKK1 inhibition of LRP6 is independent of LRP6 internalization and degradation [19]. DKK1 plays essential roles in anterio-posterior patterning, limb development, somitogenesis, eye formation and cardiogenesis [20, 30]. DKK1 knockout mice die at birth, lack anterior head structures and have forelimb and hind limb malformations [31]. Heterozygous DKK1 ($DKK1^{+/-}$) mutant mice are viable, but displays a high bone mass phenotype due to increased numbers of osteoblasts and bone formation rate [32]. Transgenic expression of DKK1 caused osteopenia with limb deformities[33], inhibited proliferation in small intestine and colon, accompanied by progressive architectural degeneration with the loss of crypts and villi [34, 35]. Thus, DKK1 is a potent negative regulator of bone formation [36] and maintains intestinal homeostasis.

DKK1 in cancer

Dysregulated activation of the Wnt signaling pathway in healthy cells can be catastrophic and is thought to play a causative role in several cancers [37]. Such activation can occur due to loss of function mutations in negative regulators of the pathway or due to gain of function or constitutive activation of positive regulators. For example, inactivating mutations in adenomatous polyposis coli (APC) or degradation resistant β-catenin in colon cancer [38]. Thus, understanding the interplay of Wnt agonists and antagonists is critical to derive a full understanding of this pathway. However, an important but less highlighted way to dysregulate Wnt signaling is the downregulation or inactivation of the secreted Wnt pathway antagonists. DKK1 is one such antagonist that must be precisely regulated and hence it could play an important role in tumor growth and progression.

DKK1, a bad molecule

DKK1 levels are elivated in a wide variety of cancers. Several reports associate DKK1 expression with progression of cancer and a poor prognosis. DKK1 overexpression has been reported in multiple myeloma [39], hepatoblastomas, Wilms' tumors [40] and breast cancer (particularly in hormone-resistant breast cancer, in familial cases and in primary tumors from patients with invaded axillary nodes) [41]. DKK1 expression in myeloma cells plays a major role in decreased bone formation seen in patients with multiple myeloma [39, 42]. DKK1 inhibits osteoblastic differentiation and high circulating levels of DKK1 in patients with multiple myeloma are associated with osteolytic lesions [43, 44].

Hall *et al*. have shown that inhibition of DKK1 expression in osteolytic PC-3 prostate cancer cells induced osteoblastic activity and, conversely, the induction of DKK1 in the mixed

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osteoblastic/osteolytic prostate cancer C4-2B cell line results in experimental bone metastases with an osteolytic phenotype [45]. In clinical specimens from prostate cancer patients, it was observed that DKK1 expression increases early in prostate cancer development and decreases during progression [46]. It is also reported that human osteolytic breast cancer cell lines produce DKK1 suggesting a role of this factor in osteolytic bone lesions from breast cancer [47]. Notably, serum DKK1 was significantly increased in women presenting with bone metastases [48]. Studies on lung and esophageal carcinomas have implied the possibility that DKK1 could be a serologic prognostic biomarker indicative of poor outcome [49].

DKK1, a good molecule

In apparent contradiction to the reports stated above, in several studies, DKK1 shows tumor growth inhibitory effects. Qiao *et al* showed that conditioned media from human mesenchymal stem cells (Z3), which have high levels of DKK1, is capable of inhibiting growth of human MCF-7 breast cancer cells *in vitro* and *in vivo* by inhibiting Wnt signaling and downregulating β-catenin [50]. Removal of DKK1 from the medium by addition of a neutralizing antibody nullified these inhibitory effects. This indicates that DKK1 is capable of growth inhibition in a paracrine fashion. There are several reports of DKK1 upregulation or over-expression causing suppressed tumor growth [51-56]. Conversely, downregulation or loss of DKK1 expression has been documented in a number of clinical studies involving breast cancer [57], melanoma [58] and colon cancer [26]. Even in xenograft studies, tumors that arose after injection of DKK1 expressing cells invariably showed a near complete loss of ectopic DKK1 expression [59]. Therefore, loss of DKK1 expression may play a role in development and/or progression of cancer. This suggests that DKK1 may have tumor suppressor functions.

A simple explanation for this activity of DKK1 is that oncogenic Wnt/β-catenin signaling is downregulated by DKK1. Interestingly, multiple reports document that DKK1 is an apoptosis inducer. DKK1 sensitizes HeLa cells to apoptosis on UV treatment [59]. Ectopically expressed DKK1 induced apoptosis in β-catenin deficient mesothelioma cell lines [60]. Shou *et al*. found that exposure of human glioma cells to DNA damaging agents caused a significant increase in DKK1 expression in a p53-independent manner. Stable expression of DKK1 into the human glioblastoma cell line (U87MG, which had undetectable levels of DKK1) increased their sensitivity to apoptosis upon DNA damage. This data supports that DKK1 is a pro-apoptotic gene and may function to amplify or transduce the pro-apototic signal following DNA damage [61]. Supporting observations were made regarding activation of DKK1 in melanoma cells. This activation causes apoptosis *in vivo* and inhibits tumor growth in nude mice. Tumors obtained in MDA-MB-435 cells when made to overexpress DKK1 showed activation of cell death and necrosis compared to the control tumors [62]. Ectopic expression of DKK1 in breast cancer cells (MDA-MB-231, T47D) caused an increased sensitivity to apoptosis along with a change in cell phenotype [57]. Bafico *et al*. analyzed the effects of DKK1 on apoptosis in MDA-MB-157 cells after oxidative stress using increasing concentrations of tert-butyl hydroperoxide and found that there was an increase in the level of apoptosis [63]. DKK1 is lost in human placental choriocarcinoma cells. Reintroduction of DKK1 in these cells caused decreased proliferation and induced apoptosis, indicating DKK1 has tumor suppressive and apoptosis inducing effects [64].

Certainly, a clearer understanding of events upstream and downstream of DKK1 expression is warranted to better appreciate functions of DKK1....

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What regulates DKK1?

One interesting and well studied fact is that DKK1 is a downstream target of Wnt signaling. This allows for a negative feedback loop. Gonzalez-Sancho *et al*. showed that activation of the canonical Wnt signaling (but not non-canonical Wnt signaling) causes an increase in transcript and protein levels of DKK1 [26]. DKK1 is a TCF target gene. The DKK1 promoter has two functional TCF binding elements and is transactivated by the β-catenin/TCF complex [65, 66]. Thus though Wnt ligands are able to signal in an autocrine manner to enhance Wnt signaling, activation of the Wnt signaling pathway ultimately leads to transcription and translation of DKK1, which in turn, inhibits Wnt signaling, establishing a negative feedback loop, thus keeping dysregulated Wnt signaling in check.. This suggests that DKK1 expression is upregulated in tumor cells in which β-catenin mediated transcription is upregulated by loss of function mutations in APC or axin or stabilization of β-catenin. DKK1 is overexpresssed in a large fraction of human hepatoblastomas, which display unregulated activation of Wnt signaling [40, 67].

DKK1 is subject to epigenetic regulation and silencing. In colon cancer cell lines, DKK1 is transcriptionally silenced by promoter hypermethylation. DKK1 was found to be hypermethylated in 17% of primary colorectal tumors [68]. In cervical cancer cell lines, DKK1 was silenced by both CpG island hypermethylation and histone deacetylation [69]. DKK1 expression is reduced in gastrointestinal cancer cell lines and cancer tissues and this correlated with CpG island hypermethylation of DKK1[70]. DKK1 gene methylation was higher (52%) in renal cell carcinoma tissues compared to adjacent normal tissues (10%) and increased methylation was associated with higher pathological stages. Renal cell carcinoma cell lines had low levels of DKK1 compared to a normal kidney cell line. Treatment of these cells with 5-Aza-2-deoxycytidine alone and with trichostatin increased DKK1 levels and apoptotic population, thus highlighting the relevance of epigenetic regulation of DKK1 [54].

Another regulator of DKK1 is vitamin D3. Aguilera *et al*. have shown that 1α,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$], the most active vitamin D metabolite, increases the level of DKK1 transcript and protein in human colon cancer cells. The effect required the presence of a transcription-competent nuclear vitamin D receptor (VDR). Thus DKK1 induction may be a mechanism through which vitamin D inhibits Wnt signaling and mediates anti-tumor activity [71]. Studies from our group have revealed that NMI (N-Myc interactor), WFDC1 (WAP four disulfide core domain 1) and Hsp40 family member DNAJB6, all of which have anti-malignant activity in melanoma and breast cancer, are activators of DKK1 gene expression [51, 52, 56]. Thus a diverse set of signaling mechanisms, tumor promoting as well as tumor suppressing, can regulate DKK1 expression. Similarly, several signaling options are evident as downstream effects of DKK1 actions.

What does DKK1 regulate?

Activities of DKK1 are not just confined to canonical Wnt/β-catenin signaling. DKK1 has been advocated to be a potential regulator of non-canonical Wnt signaling as well. It has been observed that DKK1 is capable of mediating its affects in a manner independent of β-catenin dependent transcription [59]. In some cases, even though DKK1 was able to inhibit β-catenin mediated transcription, no effects were observed on downstream targets such as c-Myc or cyclin D1 expression [59, 62, 63]. Furthermore, DKK1 suppressed tumor growth even in a β-catenin deficient mesothelioma cell line, indicating that DKK1 can act through mechanisms independent of those mediated through canonical Wnt signaling [60]. Activation of LRP6, a DKK1 receptor, by Wnt ligands may have effects that are independent of β-catenin that can still be modulated by DKK1 [20, 72]. Activation of Jun N-terminal kinase (JNK) pathway could be another such β-catenin independent effect of blocking Wnt-LRP6

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signaling. It has been demonstrated that interplay of Wnt11, DKK1 and Crescent can activate JNK to mediate its tumor suppressive effects [73, 7462].

Alternatively, DKK1 may activate CamKII causing decreased tumor growth. Mikheev *et al*. showed that DKK1 overexpression increased activity of the CamKII pathway in breast cancer cells and increased the levels of PKC phosphorylation. Thus DKK1 could mediate its tumor suppressive activity independent of β-catenin dependent transcription through the non canonical Wnt/Ca^{2+} signaling pathway by activation of CamKII. However the exact mechanism by which DKK1 causes CamKII activation is unknown [57]. Another event downstream of DKK1 is reported in a study by Koppen *et al*. implicates a gene, synaptopodin 2 (SYNPO2) which encodes an actin-binding protein, as a gene upregulated by DKK1 that inhibits neuroblastoma cell proliferation and invasiveness [75].

DKK1 negatively regulates EMT

It is becoming increasingly evident that epithelial-mesenchymal transition (EMT) is one of the critical events in tumor cell invasion and metastasis. In fact, EMT has recently been recognized as one of the hallmarks of cancer [76]. The Wnt/β-catenin pathway is one of the critical pathways that regulate epithelial plasticity and EMT by regulating mesenchymal phenotype promoting transcription factors such as SNAIL [77, 78]. Hence, it is intuitive that DKK1 may negatively regulate EMT. Studies by DiMeo *et al*. showed that upon exogenous expression of DKK1 in SUM1315 (a human malignant breast cancer cell line with mesenchymal-like phenotype), EMT promoting transcription factors, SLUG and TWIST were significantly reduced [53]. Studies from our laboratory have shown that silencing DKK1 expression from MCF10A, a non tumorigenic-epithelial breast cell line led to increased invasive capacity and decreased E-cadherin expression [51]. As seen **Figure 1** upon treatment with DKK1 protein, SUM1315 shows dramatic cell shape changes in 3D culture, pointing towards a net negative effect of DKK1 on EMT.

DKK1 contributes to the microenvironment

The role of DKK1 in cancer is diverse. Since DKK1 is a negative regulator of bone formation, patient outcome can be positive or negative with respect to DKK1 levels, especially for cancers which have some involvement with bone. A number of clinical studies have shown that DKK1 is elevated in the serum of cancer patients as compared to normal controls. As a secreted protein, DKK1 could be a key factor in determining the tumor microenvironment. Hall *et al*. suggest that DKK1 may act as a switch that transitions prostate cancer bone metastases from osteolytic to osteoblastic based on cell type and microenvironment [79, 80].

Autocrine Wnt signaling is observed in around 25% of human breast and ovarian cell lines. This signaling may result from Wnt ligand misexpression, aberrant upregulation of some other components such as a specific co-receptor not normally present, or downregulation of Wnt antagonists [63].

Could the increased levels of DKK1 still be tumor protective when observed in high levels in the serum of patients? We know that DKK1 is a downstream target of β-catenin mediated transcriptional activity. If β-catenin mediated transcription is not precisely regulated then its downstream target genes will be aberrantly expressed. Excessive DKK1 may accumulate but will still not be able to exercise control of the pathway due to malfunction of some component downstream of DKK1. Thus even though DKK1 is not responsible for tumor growth, increased levels are observed in patients with cancer, possibly as a side effect of increased β-catenin activity. However, if DKK1 is upregulated in a paracrine fashion, it will have bystander suppressive effect on Wnt signaling of the surrounding cells that may still be

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intact for components of regulated Wnt/β-catenin signaling. This may have a growth suppressive effect on those cells. However, the cells with dysregulated Wnt/β-catenin signaling and impaired for signaling downstream of DKK1 may eventually outgrow.

It is conceivable that when this elevated DKK1 reaches sites where it normally functions, for example, the bone, it will disrupt the normal resting state balance and lead to osteolytic lesions, this may also be a potential way of conditioning the metastatic niche. Currently such hypotheses are speculative but certainly offer an explanation for the confounding and sometimes overtly conflicting reports of the role of DKK1 in normal physiology and cancer. Also, different types of cancer and different stages of cancer, dependent on the tissue of origin (epithelial or mesenchymal) or cellular subtypes, may be intrinsically driven by differing effects of the Wnt/beta-catenin pathway. One cannot ignore the role that β-catenin plays as an epithelial molecule and total absence of that may in some cases drive invasive or metastatic phenotype. It must also be noted that DKK1 is a secreted glycoprotein. Thus a difference in secondary modifications on DKK1 protein or change in relative abundance of its ligands and their secondary modifications could impact its activities. However, studies specifically addressing secondary modifications of DKK1 and their functional roles are lacking. A possibility still remains that DKK1 might bind proteins other than LRP5/6 or KREMEN and have distinct, still unknown mechanisms of action. Given the dichotomy in the activity of DKK1, it is imperative to understand the mechanistic and signaling implications by addressing and exploring these different possibilities. In summary, DKK1 could have a cancer type specific and patient specific role that is critical to determining the tumor microenvironment.

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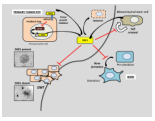

Figure 1.
DKK1 mediates cellular cross talk and impacts the cellular microenvironment. It is produced by tumor cells and other cell types such as mesenchymal stem cells (MSC). Its presence promotes apoptosis and can inhibit tumor growth, bone formation and MSC self renewal. EMT is also inhibited by DKK1. Inset photomicrographs show effect of DKK1 on the 3D morphology of SUM1315 breast cancer cells. In absence of DKK1 these mesenchymal-like cells display highly invasive outgrowths (marked by arrowheads) while in the presence of DKK1 they transform into closely packed spherical structures.

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