WNT ANTAGONISTS AND THEIR USE IN THE DIAGNOSIS AND TREATMENT OF WNT-MEDIATED DISORDERS

RELATED APPLICATIONS

This application claims priority to and the benefit of United States Provisional Application Serial No. 60/825,063, filed September 8, 2006, and United States Provisional Application Serial No. 60/951,175, filed July 20, 2007, both of which are incorporated by reference in their entirety herein.

FIELD OF THE INVENTION

The present invention relates generally to the regulation of cell growth. More specifically, the present invention relates to inhibitors of the Wnt pathway as well as to their use in the diagnosis and treatment of disorders characterized by the activation of Wnt pathway signaling, as well as to the modulation of cellular events mediated by Wnt pathway signaling.

BACKGROUND OF THE INVENTION

The Wnt signaling pathway's association with carcinogenesis began as a result of early observations and experiments in certain murine mammary tumors. Wnt-1 proto-oncogene (Int-1) was originally identified from mammary tumors induced by mouse mammary tumor virus (MMTV) due to an insertion of a viral DNA sequence. Nusse *et al.*, *Cell* 1982; **31**: 99-109. The result of such viral integration was unregulated expression of Int-1 resulting in the formation of tumors. Vanooyen, A. *et al.*, *Cell* 1984; **39**: 233-240; Nusse, R. *et al., Nature* 1984; **307**: 131-136; Tsukamoto *et al., Cell* 1988; **55**: 619-625. Subsequent sequence analysis demonstrated that the Int-1 was a mammalian homolog of the Drosophila gene Wingless (Wg), which was implicated in development, and the terms were then combined to create "Wnt" to identify this family of proteins.

The human Wnt gene family of secreted ligands has now grown to at least 19 members (e.g., Wnt-1 (RefSeq.: NM_005430), Wnt-2 (RefSeq.: NM_003391), Wnt-2B (Wnt-13) (RefSeq.: NM_004185), Wnt-3 (ReSeq.: NM_030753), Wnt3a (RefSeq.: NM_033131), Wnt-4 (RefSeq.: NM_030761), Wnt-5A (RefSeq.: NM_003392), Wnt-5B (RefSeq.: NM_032642), Wnt-6 (RefSeq.: NM_006522), Wnt-7A (RefSeq.: NM_004625), Wnt-7B (RefSeq.: NM_058238), Wnt-8A (RefSeq.: NM_058244), Wnt-8B (RefSeq.:

NM_003393), Wnt-9A (Wnt-14) (RefSeq.: NM_003395), Wnt-9B (Wnt-15) (RefSeq.: NM_003396), Wnt-10A (RefSeq.: NM_025216), Wnt-10B (RefSeq.: NM_003394), Wnt-11 (RefSeq.: NM_004626), Wnt-16 (RefSeq.: NM_016087)). Each member has varying degrees of sequence identity but all contain 23-24 conserved cysteine residues which show highly conserved spacing. McMahon, AP *et al., Trends Genet.* 1992; **8**: 236-242; Miller, JR. *Genome Biol.* 2002; **3(1)**: 3001.1-3001.15. The Wnt proteins are small (i.e., 39-46 kD) acylated, secreted glycoproteins which play key roles in both embryogenesis and mature tissues. During embryological development, the expression of Wnt proteins is important in patterning through control of cell proliferation and determination of stem cell fate. The Wnt molecules are also palmitoylated, and thus are more hydrophobic than would be otherwise predicted by analysis of the amino acid sequence alone. Willert, K. *et al., Nature* 2003; **423**: 448-52. The site or sites of palmitoylation are also believed to be essential for function.

The Wnt proteins act as ligands to activate the Frizzled (Frz) family of seven-pass transmembrane receptors. Ingham, P.W. *Trends Genet.* 1996; **12**: 382-384; YangSnyder, J. *et al., Curr. Biol.* 1996; **6**: 1302-1306; Bhanot, P. *et al., Nature* 1996; **382**: 225-230. There are ten known members of the Frz family (e.g., Frz1, Frz2, Frz3 . . . Frz10), each characterized by the presence of a cysteine rich domain (CRD). Huang et al., *Genome Biol.* 2004; **5**: 234.1-234.8. There is a great degree of promiscuity between the various Wnt-Frizzled interactions, but Wnt-Frz binding must also incorporate the LDL receptor related proteins (LRP5 or LRP6) and the membrane and the cytoplasmic protein Dishevelled (Dsh) to form an active signaling complex.

The binding of Wnt to Frizzled can activate signaling via either the canonical Wnt signaling pathway, thereby resulting in stabilization and increased transcriptional activity of β-catenin [Peifer, M. *et al., Development* 1994; **120**: 369-380; Papkoff, J. *et al., Mol. Cell Biol.* 1996; **16**: 2128-2134] or non-canonical signaling, such as through the Wnt/planar cell polarity (Wnt/PCP) or Wnt-calcium (Wnt/Ca^{2+}) pathway. Veeman, M.T. *et al., Dev. Cell* 2003; 5: 367-377.

The canonical Wnt signaling pathway is the most relevant of the Wnt signaling pathways to the development of cancer. Ilyas, M. *J. Pathol.* 2005; **205**: 130-144. Normal activation of this pathway begins a series of downstream events culminating in the stabilization and increased levels of the protein β-catenin. This protein is normally an inactive cytoplasmic protein, and is found at the cell membrane bound to proteins including e-cadherin. In the absence of Wnt ligand, phosphorylated cytoplasmic β-catenin is normally

rapidly degraded. Upon activation of the canonical pathway, unphosphorylated β-catenin is transported to the nucleus where it further results in transcriptional activation of various target genes. The subsequent upregulation in transcription of these target genes leads to changes in the cell, and continuous, unregulated expression of such target genes results in tumor development. Since aberrant Wnt signaling appears to be a necessary precursor in carcinogenesis, effective inhibitors of Wnt signaling are of great interest as cancer therapeutics.

The use of soluble receptors as antagonists to ligand-receptor interactions is known in the art. Such molecules can be effective therapeutic antagonists if they bind the free ligand in a manner so as to prevent the initial receptor activation step of the signaling pathway. Soluble minimal extracellular domain (ECD) fragments of the cysteine-rich domain (CRD) of a Frizzled receptor which exhibit binding to Wnt have been identified, based on crystallography data. Dann *et al., Nature* 412: 86-90 (2001). However, while such Frizzled fragments did exhibit binding to Wnt ligand, such fragments are unsuitable for therapeutics because of their rapid degradation *in vivo*.

The use of a soluble Frizzled domain coupled to an immunoglobulin Fc as a potential Wnt antagonist has been proposed. Therapeutic Opportunities of the Wnt Signaling Pathway in Cancer, New York Academy of Sciences, Oct. 25, 2005; Hsieh, J-C. et al., *PNAS*, 96: 3546-3551 (1999). However, prior to the present invention, attempts at creating a soluble Frizzled receptor-Fc fusion therapeutic were not successful. For example, one such chimera based on residues 1-173 of the Frz8 CRD (Frz (173)-Fc, SEQ ID NO: 113) had suboptimal efficacy (Figure 12), and was unstable *in vivo* (Figure 11). Moreover, the Frz (173)-Fc chimera only reduced the rate of increase in tumor volume (as opposed to shrinking starting tumor volume). Additionally, while the creation of Fc fusions is generally known as one technique to improve the *in vivo* stability of the resulting construct, the creation of effective therapeutic Fc constructs can be difficult owing to a number of problems, including improper protein folding of the new protein construct and steric hindrance of the fusion construct to the target.

Thus, a need exits for a Wnt antagonist therapeutic with enhanced *in vivo* stability that acts to inhibit Wnt ligand induced cellular signaling.

SUMMARY OF THE INVENTION

The invention provides for compositions and their use in methods of diagnosing and treating Wnt-mediated disorder, such as cancer, and in inhibiting cellular

Wnt signaling. Specifically, the invention provides for Wnt antagonists that are chimeric molecules comprising a Frizzled domain component, such as a polypeptide derived from a Frizzled (Frz) protein, a Frizzled related protein (sFRP) or another protein (e.g., Ror-1, -2, etc.), and an immunoglobulin Fc domain, and their use in methods of diagnosing and treating Wnt-mediated disorders and in inhibiting cellular Wnt signaling.

One aspect of the invention provides for a Wnt antagonist comprising a Frizzled domain component and a Fc domain. The Frizzled domain component of the Wnt antagonist comprises a polypeptide derived from a Frz protein, a FRP protein, or a Ror protein. In one embodiment, the Wnt antagonist is active *in vivo* for at least 1 hour. In another embodiment, the Wnt antagonist is active *in vivo* for at least 5 hours. In another embodiment, the Wnt antagonist has an *in vivo* half-life of at least 1 day. In yet another embodiment, the Wnt antagonist has an *in vivo* half-life of at least 2 days.

In a further embodiment, the Frizzled domain component comprises a minimal CRD (ECD) domain from a Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 18), hFrz2 (SEQ ID NO: 19), hFrz3 (SEQ ID NO: 20), hFrz4 (SEQ ID NO: 21), hFrz5 (SEQ ID NO: 22), hFrz6 (SEQ ID NO: 23), hFrz7 (SEQ ID NO: 24), hFrz8 (SEQ ID NO: 25), hFrz9 (SEQ ID NO: 26), and hFrz10 (SEQ ID NO: 27), and active variants thereof. In yet a further embodiment, the Frizzled domain component comprises a minimal CRD (ECD) domain from a sFRP polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 28), sFRP2 (SEQ ID NO: 29), sFRP3 (SEQ ID NO: 30), sFRP4 (SEQ ID NO: 31), and sFRP5 (SEQ ID NO: 32), and active variants thereof. In yet a further embodiment, the Frizzled domain component comprises a minimal CRD (ECD) domain from a Ror polypeptide selected from the group consisting of hRor1 (SEQ ID NO: 33), and hRor2 (SEQ ID NO: 34), and active variants thereof.

In yet a further embodiment, the Frizzled domain component comprises a mature Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 50), hFrz2 (SEQ ID NO: 51), hFrz3 (SEQ ID NO: 52), hFrz4 (SEQ ID NO: 53), hFrz5 (SEQ ID NO: 54), hFrz6 (SEQ ID NO: 55), hFrz7 (SEQ ID NO: 56), hFrz8 (SEQ ID NO: 57), hFrz9 (SEQ ID NO: 58), and hFrz10 (SEQ ID NO: 59), and active variants thereof, or a mature sFrp polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 60), sFRP2 (SEQ ID NO: 61), sFRP3 (SEQ ID NO: 62), sFRP4 (SEQ ID NO: 63), and sFRP5 (SEQ ID NO: 64), and active variants thereof, or a mature Ror polypeptide selected from the group consisting of hRor1 (SEQ ID NO: 65), and hRor2 (SEQ ID NO: 66), and active variants thereof.

In a still further embodiment, the Frizzled domain component comprises a pro-Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 35), hFrz2 (SEQ ID NO: 36), hFrz3 (SEQ ID NO: 37), hFrz4 (SEQ ID NO: 38), hFrz5 (SEQ ID NO: 39), hFrz6 (SEQ ID NO: 40), hFrz7 (SEQ ID NO: 41), hFrz8 (SEQ ID NO: 42), hFrz9 (SEQ ID NO: 43), and hFrz10 (SEQ ID NO: 44), and active variants thereof, or a pro-sFrp polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 45), sFRP2 (SEQ ID NO: 46), sFRP3 (SEQ ID NO: 47), sFRP4 (SEQ ID NO: 48), and sFRP5 (SEQ ID NO: 49), and active variants thereof.

In one embodiment, the Wnt antagonist comprises a Fc component derived from an immunoglobulin selected from the group consisting of IgG1, IgG2, IgG3 and IgG4. In another embodiment, the Fc is derived from an IgG1 immunoglobulin. In yet another embodiment the Fc sequence comprises the Fc shown in SEQ ID NO: 67 or SEQ ID NO: 68.

In one embodiment, the Wnt antagonist further comprises a linker connecting the Frizzled domain component to the Fc domain. In one such embodiment, the linker is a peptide linker such as ESGGGGVT (SEQ ID NO: 69), LESGGGGVT (SEQ ID NO: 70), GRAQVT (SEQ ID NO: 71), WRAQVT (SEQ ID NO: 72), and ARGRAQVT (SEQ ID NO: 73).

In particular embodiments, the Wnt antagonist comprises a polypeptide selected from the group consisting of in Frz8-Fc (SEQ ID NO: 74), Frz5-Fc (SEQ ID NO: 75), Frz1-Fc (SEQ ID NO: 76), Frz2-Fc (SEQ ID NO: 77), Frz3-Fc (SEQ ID NO: 78), Frz4-Fc (SEQ ID NO: 79), Frz6-Fc (SEQ ID NO: 80), Frz7-Fc (SEQ ID NO: 81), Frz9-Fc (SEQ ID NO: 82), Frz10-Fc (SEQ ID NO: 83), sFRP1-Fc (SEQ ID NO: 84), sFRP2 (SEQ ID NO: 85), sFRP3-Fc (SEQ ID NO: 86), sFRP4-Fc (SEQ ID NO: 87), and sFRP5-Fc (SEQ ID NO: 88).

Another aspect of the invention provides for a composition comprising at least one pharmaceutically acceptable carrier or excipient and a Wnt antagonist as described above.

Yet another aspect of the invention provides for a nucleic acid sequence encoding any of the Wnt antagonists described above. In one embodiment, the nucleic acid encoding a Wnt antagonist further comprises a vector containing control sequences to which the nucleic acid is operably linked. In another embodiment, the vector is contained in host cells, such as a mammalian, insect, *E. coli* or yeast cell.

Another aspect of the invention provides for an article of manufacture comprising a composition comprising at least one pharmaceutically acceptable carrier or excipient and a Wnt antagonist as described above and a container, wherein the Wnt antagonist is contained

within the container and the container further comprises (a) a label affixed to the container, or (b) a package insert inside the container referring to the use of the Wnt antagonist indicating use of the composition for the therapeutic treatment or diagnostic detection of a Wnt-mediated disorder.

Yet another aspect of the invention provides for a method of inhibiting Wnt signaling in a cell comprising contacting the cell with an effective amount of a Wnt antagonist comprising a Frizzled domain component and a Fc domain. The Frizzled domain component of the Wnt antagonist comprises a polypeptide derived from a Frz protein, a FRP protein, or a Ror protein. In one embodiment, the Wnt antagonist is active *in vivo* for at least 1 hour. In another embodiment, the Wnt antagonist is active *in vivo* for at least 5 hours. In another embodiment, the Wnt antagonist has an *in vivo* half-life of at least 1 day. In yet another embodiment, the Wnt antagonist has an *in vivo* half-life of at least 2 days.

In a further embodiment of this aspect, the Frizzled domain component comprises a minimal CRD (ECD) domain from a Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 18), hFrz2 (SEQ ID NO: 19), hFrz3 (SEQ ID NO: 20), hFrz4 (SEQ ID NO: 21), hFrz5 (SEQ ID NO: 22), hFrz6 (SEQ ID NO: 23), hFrz7 (SEQ ID NO: 24), hFrz8 (SEQ ID NO: 25), hFrz9 (SEQ ID NO: 26), and hFrz10 (SEQ ID NO: 27), and active variants thereof. In yet a further embodiment, the Frizzled domain component comprises a minimal CRD (ECD) domain from a sFRP polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 28), sFRP2 (SEQ ID NO: 29), sFRP3 (SEQ ID NO: 30), sFRP4 (SEQ ID NO: 31), and sFRP5 (SEQ ID NO: 32), and active variants thereof. In yet a further embodiment, the Frizzled domain component comprises a minimal CRD (ECD) domain from a Ror polypeptide selected from the group consisting of hRor1 (SEQ ID NO: 33), and hRor2 (SEQ ID NO: 34), and active variants thereof.

In yet a further embodiment, the Frizzled domain component comprises a mature Frz polypeptide selected from the group consisting of: (SEQ ID NO: 50), hFrz2 (SEQ ID NO: 51), hFrz3 (SEQ ID NO: 52), hFrz4 (SEQ ID NO: 53), hFrz5 (SEQ ID NO: 54), hFrz6 (SEQ ID NO: 55), hFrz7 (SEQ ID NO: 56), hFrz8 (SEQ ID NO: 57), hFrz9 (SEQ ID NO: 58), and hFrz10 (SEQ ID NO: 59), and active variants thereof, or a mature sFrp polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 60), sFRP2 (SEQ ID NO: 61), sFRP3 (SEQ ID NO: 62), sFRP4 (SEQ ID NO: 63), and sFRP5 (SEQ ID NO: 64), and active variants thereof, or a mature Ror polypeptide selected from the group consisting of hRor1 (SEQ ID NO: 65), and hRor2 (SEQ ID NO: 66), and active variants thereof.

In a still further embodiment, the Frizzled domain component comprises a pro-Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 35), hFrz2 (SEQ ID NO: 36), hFrz3 (SEQ ID NO: 37), hFrz4 (SEQ ID NO: 38), hFrz5 (SEQ ID NO: 39), hFrz6 (SEQ ID NO: 40), hFrz7 (SEQ ID NO: 41), hFrz8 (SEQ ID NO: 42), hFrz9 (SEQ ID NO: 43), and hFrz10 (SEQ ID NO: 44), and active variants thereof, or a pro-sFrp polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 45), sFRP2 (SEQ ID NO: 46), sFRP3 (SEQ ID NO: 47), sFRP4 (SEQ ID NO: 48), and sFRP5 (SEQ ID NO: 49), and active variants thereof.

In one embodiment, the Wnt antagonist comprises a Fc component derived from an immunoglobulin selected from the group consisting of IgG1, IgG2, IgG3 and IgG4. In another embodiment, the Fc is derived from an IgG1 immunoglobulin. In yet another embodiment the Fc sequence shown in SEQ ID NO: 67 or SEQ ID NO: 68.

In one embodiment, Wnt antagonist further comprises a linker connecting the Frizzled domain component to the Fc domain. In one embodiment, the linker is a peptide linker such as ESGGGGVT (SEQ ID NO: 69), LESGGGGVT (SEQ ID NO: 70), GRAQVT (SEQ ID NO: 71), WRAQVT (SEQ ID NO: 72), and ARGRAQVT (SEQ ID NO: 73).

In particular embodiments, the Wnt antagonist comprises a polypeptide selected from the group consisting of Frz8-Fc (SEQ ID NO: 74), Frz5-Fc (SEQ ID NO: 75), Frz1-Fc (SEQ ID NO: 76), Frz2-Fc (SEQ ID NO: 77), Frz3-Fc (SEQ ID NO: 78), Frz4-Fc (SEQ ID NO: 79), Frz6-Fc (SEQ ID NO: 80), Frz7-Fc (SEQ ID NO: 81), Frz9-Fc (SEQ ID NO: 82), Frz10-Fc (SEQ ID NO: 83), sFRP1-Fc (SEQ ID NO: 84), sFRP2 (SEQ ID NO: 85), sFRP3-Fc (SEQ ID NO: 86), sFRP4-Fc (SEQ ID NO: 87), and sFRP5-Fc (SEQ ID NO: 88).

In one embodiment of this method, the cell is contained within a mammal and the amount administered is a therapeutically effective amount. In another embodiment, the Wnt signaling results from activation of a Wnt signaling component through somatic mutation. In another embodiment, the inhibition of Wnt signaling results in the inhibition of growth of the cell. In yet another embodiment, the cell is a cancer cell.

Another aspect of the invention provides for a method of treating a Wnt-mediated disorder in a mammal suffering therefrom, comprising administering to the mammal a therapeutically effective amount of a Wnt antagonist comprising a Frizzled domain component and a Fc domain. The Frizzled domain component of the Wnt antagonist comprises a polypeptide derived from a Frz protein, a FRP protein, or a Ror protein. In one embodiment, the Wnt antagonist is active *in vivo* for at least 1 hour. In another embodiment,

the Wnt antagonist is active *in vivo* for at least 5 hours. In another embodiment, the Wnt antagonist has an *in vivo* half-life of at least 1 day. In yet another embodiment, the Wnt antagonist has an *in vivo* half-life of at least 2 days.

In a further embodiment of this aspect, the Frizzled domain component comprises a minimal CRD (ECD) domain from a Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 18), hFrz2 (SEQ ID NO: 19), hFrz3 (SEQ ID NO: 20), hFrz4 (SEQ ID NO: 21), hFrz5 (SEQ ID NO: 22), hFrz6 (SEQ ID NO: 23), hFrz7 (SEQ ID NO: 24), hFrz8 (SEQ ID NO: 25), hFrz9 (SEQ ID NO: 26), and hFrz10 (SEQ ID NO: 27), and active variants thereof. In yet a further embodiment, the Frizzled domain component comprises a minimal CRD (ECD) domain from a sFRP polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 28), sFRP2 (SEQ ID NO: 29), sFRP3 (SEQ ID NO: 30), sFRP4 (SEQ ID NO: 31), and sFRP5 (SEQ ID NO: 32), and active variants thereof. In yet a further embodiment, the Frizzled domain component comprises a minimal CRD (ECD) domain from a Ror polypeptide selected from the group consisting of hRor1 (SEQ ID NO: 33), and hRor2 (SEQ ID NO: 34), and active variants thereof.

In yet a further embodiment, the Frizzled domain component comprises a mature Frz polypeptide selected from the group consisting of: (SEQ ID NO: 50), hFrz2 (SEQ ID NO: 51), hFrz3 (SEQ ID NO: 52), hFrz4 (SEQ ID NO: 53), hFrz5 (SEQ ID NO: 54), hFrz6 (SEQ ID NO: 55), hFrz7 (SEQ ID NO: 56), hFrz8 (SEQ ID NO: 57), hFrz9 (SEQ ID NO: 58), and hFrz10 (SEQ ID NO: 59), and active variants thereof, or a mature sFrp polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 60), sFRP2 (SEQ ID NO: 61), sFRP3 (SEQ ID NO: 62), sFRP4 (SEQ ID NO: 63), and sFRP5 (SEQ ID NO: 64), and active variants thereof, or a mature Ror polypeptide selected from the group consisting of hRor1 (SEQ ID NO: 65), and hRor2 (SEQ ID NO: 66), and active variants thereof.

In still further embodiments, the Frizzled domain component comprises a pro-Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 35), hFrz2 (SEQ ID NO: 36), hFrz3 (SEQ ID NO: 37), hFrz4 (SEQ ID NO: 38), hFrz5 (SEQ ID NO: 39), hFrz6 (SEQ ID NO: 40), hFrz7 (SEQ ID NO: 41), hFrz8 (SEQ ID NO: 42), hFrz9 (SEQ ID NO: 43), and hFrz10 (SEQ ID NO: 44), and active variants thereof, or a pro-sFrp polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 45), sFRP2 (SEQ ID NO: 46), sFRP3 (SEQ ID NO: 47), sFRP4 (SEQ ID NO: 48), and sFRP5 (SEQ ID NO: 49), and active variants thereof.

In one embodiment, the Wnt antagonist comprises a Fc component derived from an immunoglobulin selected from the group consisting of IgG1, IgG2, IgG3 and IgG4. In another embodiment, the Fc is derived from an IgG1 immunoglobulin. In yet another embodiment the Fc sequence shown in SEQ ID NO: 67 or SEQ ID NO: 68.

In one embodiment, Wnt antagonist further comprises a linker connecting the Frizzled domain component to the Fc domain. In one embodiment, the linker is a peptide linker such as ESGGGGVT (SEQ ID NO: 69), LESGGGGVT (SEQ ID NO: 70), GRAQVT (SEQ ID NO: 71), WRAQVT (SEQ ID NO: 72), and ARGRAQ VT (SEQ ID NO: 73).

In particular embodiments, the Wnt antagonist comprises a polypeptide selected from the group consisting of Frz8-Fc (SEQ ID NO: 74), Frz5-Fc (SEQ ID NO: 75), Frz1-Fc (SEQ ID NO: 76), Frz2-Fc (SEQ ID NO: 77), Frz3-Fc (SEQ ID NO: 78), Frz4-Fc (SEQ ID NO: 79), Frz6-Fc (SEQ ID NO: 80), Frz7-Fc (SEQ ID NO: 81), Frz9-Fc (SEQ ID NO: 82), Frz10-Fc (SEQ ID NO: 83), sFRP1-Fc (SEQ ID NO: 84), sFRP2 (SEQ ID NO: 85), sFRP3-Fc (SEQ ID NO: 86), sFRP4-Fc (SEQ ID NO: 87), and sFRP5-Fc (SEQ ID NO: 88).

In one embodiment of this method, the disorder is a cell proliferative disorder associated with aberrant Wnt signaling activity. In another embodiment, the aberrant Wnt signaling activity results from increased expression of a Wnt protein. In yet another embodiment, the cell proliferative disorder is cancer, such as of colon cancer, colorectal cancer, breast cancer, leukemia, gliomas, or medulloblastomas.

Yet another aspect of the invention provides for a method for detecting the presence of a Wnt protein, comprising contacting the sample with a Wnt antagonist as described above, where the presence of a complex, or the binding level between the Wnt antagonist and Wnt protein is indicative of the presence of a Wnt protein and/or signaling. In one embodiment, the method further comprises determining if the level of Wnt signaling is aberrant, the method further comprising comparing the level of binding in the sample to the level in a second sample known to have physiologically normal Wnt signaling. A level of binding in the sample that is higher or lower than that of the second sample is indicative of aberrant Wnt signaling. In yet another embodiment, the aberrant Wnt signaling is further indicative of the presence of a Wnt-mediated disorder, such as cancer.

Another aspect of the invention provides for a method of modulating the expression of a Wnt target gene in a cell characterized by activated or excessive Wnt signaling, comprising contact the cell with an effective amount of a Wnt antagonist described above.

Yet another aspect of the invention provides for a method of therapeutically treating a Wnt-mediated cancer, comprising administering a therapeutically effective amount of a Wnt antagonist comprising a Frizzled domain component and a Fc domain. The Frizzled domain component of the Wnt antagonist comprises a polypeptide derived from a Frz protein, a FRP protein, or a Ror protein. In one embodiment, the Wnt antagonist is active *in vivo* for at least 1 hour. In another embodiment, the Wnt antagonist is active *in vivo* for at least 5 hours. In another embodiment, the Wnt antagonist has an *in vivo* half-life of at least 1 day. In yet another embodiment, the Wnt antagonist has an *in vivo* half-life of at least 2 days.

In a further embodiment of this aspect, the Frizzled domain component comprises a minimal CRD (ECD) domain from a Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 18), hFrz2 (SEQ ID NO: 19), hFrz3 (SEQ ID NO: 20), hFrz4 (SEQ ID NO: 21), hFrz5 (SEQ ID NO: 22), hFrz6 (SEQ ID NO: 23), hFrz7 (SEQ ID NO: 24), hFrz8 (SEQ ID NO: 25), hFrz9 (SEQ ID NO: 26), and hFrz10 (SEQ ID NO: 27), and active variants thereof. In yet a further embodiment, the Frizzled domain component comprises a minimal CRD (ECD) domain from a sFRP polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 28), sFRP2 (SEQ ID NO: 29), sFRP3 (SEQ ID NO: 30), sFRP4 (SEQ ID NO: 31), and sFRP5 (SEQ ID NO: 32), and active variants thereof. In yet a further embodiment, the Frizzled domain component comprises a minimal CRD (ECD) domain from a Ror polypeptide selected from the group consisting of hRor1 (SEQ ID NO: 33), and hRor2 (SEQ ID NO: 34), and active variants thereof.

In yet a further embodiment, the Frizzled domain component comprises a mature Frz polypeptide selected from the group consisting of: (SEQ ID NO: 50), hFrz2 (SEQ ID NO: 51), hFrz3 (SEQ ID NO: 52), hFrz4 (SEQ ID NO: 53), hFrz5 (SEQ ID NO: 54), hFrz6 (SEQ ID NO: 55), hFrz7 (SEQ ID NO: 56), hFrz8 (SEQ ID NO: 57), hFrz9 (SEQ ID NO: 58), and hFrz10 (SEQ ID NO: 59), and active variants thereof, or a mature sFrp polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 60), sFRP2 (SEQ ID NO: 61), sFRP3 (SEQ ID NO: 62), sFRP4 (SEQ ID NO: 63), and sFRP5 (SEQ ID NO: 64), and active variants thereof, or a mature Ror polypeptide selected from the group consisting of hRor1 (SEQ ID NO: 65), and hRor2 (SEQ ID NO: 66), and active variants thereof.

In a still further embodiment, the Frizzled domain component comprises a pro-Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 35), hFrz2 (SEQ ID NO: 36), hFrz3 (SEQ ID NO: 37), hFrz4 (SEQ ID NO: 38), hFrz5 (SEQ ID NO: 39), hFrz6

(SEQ ID NO: 40), hFrz7 (SEQ ID NO: 41), hFrz8 (SEQ ID NO: 42), hFrz9 (SEQ ID NO: 43), and hFrz10 (SEQ ID NO: 44), and active variants thereof, or a pro-sFrp polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 45), sFRP2 (SEQ ID NO: 46), sFRP3 (SEQ ID NO: 47), sFRP4 (SEQ ID NO: 48), and sFRP5 (SEQ ID NO: 49), and active variants thereof.

In one embodiment, the Wnt antagonist comprises a Fc component derived from an immunoglobulin selected from the group consisting of IgG1, IgG2, IgG3 and IgG4. In another embodiment, the Fc is derived from an IgG1 immunoglobulin. In yet another embodiment the Fc sequence shown in SEQ ID NO: 67 or SEQ ID NO: 68.

In one embodiment, Wnt antagonist further comprises a linker connecting the Frizzled domain component to the Fc domain. In one embodiment, the linker is a peptide linker such as ESGGGGVT (SEQ ID NO: 69), LESGGGGVT (SEQ ID NO: 70), GRAQVT (SEQ ID NO: 71), WRAQVT (SEQ ID NO: 72), and ARGRAQVT (SEQ ID NO: 73).

In particular embodiments, the Wnt antagonist comprises a polypeptide selected from the group consisting of Frz8-Fc (SEQ ID NO: 74), Frz5-Fc (SEQ ID NO: 75), Frz1-Fc (SEQ ID NO: 76), Frz2-Fc (SEQ ID NO: 77), Frz3-Fc (SEQ ID NO: 78), Frz4-Fc (SEQ ID NO: 79), Frz6-Fc (SEQ ID NO: 80), Frz7-Fc (SEQ ID NO: 81), Frz9-Fc (SEQ ID NO: 82), Frz10-Fc (SEQ ID NO: 83), sFRP1-Fc (SEQ ID NO: 84), sFRP2 (SEQ ID NO: 85), sFRP3-Fc (SEQ ID NO: 86), sFRP4-Fc (SEQ ID NO: 87), and sFRP5-Fc (SEQ ID NO: 88).

The administration of the antagonist arrests any subsequent increase in size or advancement in severity of the cancer. In one embodiment, the administration of the Wnt antagonist results in the reduction in size or severity of the cancer. In another embodiment, the administration of the Wnt antagonist reduces the tumor burden of the cancer. In yet another embodiment, the administration of the Wnt antagonist kills the cancer.

Another aspect of the invention provides for the use of a Wnt antagonist in the manufacture of a medicament for the treatment of a cell proliferative disorder. Wnt antagonist comprises a Frizzled domain component and a Fc domain. The Frizzled domain component of the Wnt antagonist comprises a polypeptide derived from a Frz protein, a FRP protein, or a Ror protein. In one embodiment, the Wnt antagonist is active *in vivo* for at least 1 hour. In another embodiment, the Wnt antagonist is active *in vivo* for at least 5 hours. In another embodiment, the Wnt antagonist has an *in vivo* half-life of at least 1 day. In yet another embodiment, the Wnt antagonist has an *in vivo* half-life of at least 2 days.

In a further embodiment of this aspect, the Frizzled domain component comprises a minimal CRD (ECD) domain from a Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 18), hFrz2 (SEQ ID NO: 19), hFrz3 (SEQ ID NO: 20), hFrz4 (SEQ ID NO: 21), hFrz5 (SEQ ID NO: 22), hFrz6 (SEQ ID NO: 23), hFrz7 (SEQ ID NO: 24), hFrz8 (SEQ ID NO: 25), hFrz9 (SEQ ID NO: 26), and hFrz10 (SEQ ID NO: 27), and active variants thereof. In yet a further embodiment, the Frizzled domain component comprises a minimal CRD (ECD) domain from a sFRP polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 28), sFRP2 (SEQ ID NO: 29), sFRP3 (SEQ ID NO: 30), sFRP4 (SEQ ID NO: 31), and sFRP5 (SEQ ID NO: 32), and active variants thereof. In yet a further embodiment, the Frizzled domain component comprises a minimal CRD (ECD) domain from a Ror polypeptide selected from the group consisting of hRor1 (SEQ ID NO: 33), and hRor2 (SEQ ID NO: 34), and active variants thereof.

In yet a further embodiment, the Frizzled domain component comprises a mature Frz polypeptide selected from the group consisting of: (SEQ ID NO: 50), hFrz2 (SEQ ID NO: 51), hFrz3 (SEQ ID NO: 52), hFrz4 (SEQ ID NO: 53), hFrz5 (SEQ ID NO: 54), hFrz6 (SEQ ID NO: 55), hFrz7 (SEQ ID NO: 56), hFrz8 (SEQ ID NO: 57), hFrz9 (SEQ ID NO: 58), and hFrz10 (SEQ ID NO: 59), and active variants thereof, or a mature sFrp polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 60), sFRP2 (SEQ ID NO: 61), sFRP3 (SEQ ID NO: 62), sFRP4 (SEQ ID NO: 63), and sFRP5 (SEQ ID NO: 64), and active variants thereof, or a mature Ror polypeptide selected from the group consisting of hRor1 (SEQ ID NO: 65), and hRor2 (SEQ ID NO: 66), and active variants thereof.

In a still further embodiment, the Frizzled domain component comprises a pro-Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 35), hFrz2 (SEQ ID NO: 36), hFrz3 (SEQ ID NO: 37), hFrz4 (SEQ ID NO: 38), hFrz5 (SEQ ID NO: 39), hFrz6 (SEQ ID NO: 40), hFrz7 (SEQ ID NO: 41), hFrz8 (SEQ ID NO: 42), hFrz9 (SEQ ID NO: 43), and hFrz10 (SEQ ID NO: 44), and active variants thereof, or a pro-sFrp polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 45), sFRP2 (SEQ ID NO: 46), sFRP3 (SEQ ID NO: 47), sFRP4 (SEQ ID NO: 48), and sFRP5 (SEQ ID NO: 49), and active variants thereof.

In one embodiment, the Wnt antagonist comprises a Fc component derived from an immunoglobulin selected from the group consisting of IgG1, IgG2, IgG3 and IgG4. In another embodiment, the Fc is derived from an IgG1 immunoglobulin. In yet another embodiment the Fc sequence shown in SEQ ID NO: 67 or SEQ ID NO: 68.

In one embodiment, Wnt antagonist further comprises a linker connecting the Frizzled domain component to the Fc domain. In one embodiment, the linker is a peptide linker such as ESGGGGVT (SEQ ID NO: 69), LESGGGGVT (SEQ ID NO: 70), GRAQVT (SEQ ID NO: 71), WRAQVT (SEQ ID NO: 72), and ARGRAQVT (SEQ ID NO: 73).

In particular embodiments, the Wnt antagonist comprises a polypeptide selected from the group consisting of Frz8-Fc (SEQ ID NO: 74), Frz5-Fc (SEQ ID NO: 75), Frz1-Fc (SEQ ID NO: 76), Frz2-Fc (SEQ ID NO: 77), Frz3-Fc (SEQ ID NO: 78), Frz4-Fc (SEQ ID NO: 79), Frz6-Fc (SEQ ID NO: 80), Frz7-Fc (SEQ ID NO: 81), Frz9-Fc (SEQ ID NO: 82), Frz10-Fc (SEQ ID NO: 83), sFRP1-Fc (SEQ ID NO: 84), sFRP2 (SEQ ID NO: 85), sFRP3-Fc (SEQ ID NO: 86), sFRP4-Fc (SEQ ID NO: 87), and sFRP5-Fc (SEQ ID NO: 88).

In one embodiment, the cell proliferative disorder is cancer such as colon cancer, colorectal cancer, breast cancer, leukemia, gliomas, or medulloblastomas.

DESCRIPTION OF THE FIGURES

Figure 1 is an abbreviated summary of the canonical Wnt signaling pathway both in the "off" or inactive state as well as the "on" or active state.

Figure 2 is a schematic diagram representing a Frizzled extracellular domain linked to the Fc region of a human immunoglobulin domain.

Figure 3 is an alignment of the 17 known Frizzled protein extracellular domains. Figure 3A shows an alignment of the extracellular domains of the 10 pro-Frizzled proteins (SEQ ID NOs: 35-44) and the 5 pro-sFRP proteins (SEQ ID NOs: 45-49), while Figure 3B shows an alignment of the extracellular domains of 10 mature Frizzled proteins (SEQ ID NOs: 50-59), and 5 mature sFRP proteins (SEQ ID NOs: 60-64), as well as the extracellular domains of the mature Ror proteins (SEQ ID NOs: 65-66). Similar residues are boxed in gray, identical residues are indicated by asterisks. Similar residues are grouped as acidic, basic, polar and non-polar. In Figure 3B, the minimal CRD (ECD) domains are indicated between the two boxed arrowed lines (SEQ ID NOs: 18-34).

Figure 4 shows the sequences of the Frz (156)-Fc and Frz (173)-Fc chimeric constructs. Figure 4A shows the longer Frz (173)-Fc sequence (SEQ ID NO: 113). Shown in bold (i.e., first 24 N-terminal amino acid residues) is the leader signal sequence. Residues 25-27 are alanine residues that may be present or absent in the mature protein. Shown in boxed text (i.e., residues 157-173) are the additional sequences of the Frz8 receptors that distinguish the longer (Frz173) from the shorter (Frz156) chimeric constructs. The linker sequence (i.e.,

residues 174-182) is underlined, while the Fc domain sequence is shown in italics (i.e., residues 183-409). Figure 4B shows the shorter Frz (156)-Fc (SEQ ID NO: 74). In bold (i.e., first 24 N-terminal amino acid residues) is the leader signal sequence. Residues 25-27 are alanine residues that may be present or absent in the mature protein. The linker sequence (i.e., residues 157-164) is underlined, while the Fc domain sequence is shown in italics (i.e., residues 165-391).

Figure 5A-5H shows the nucleic acid sequence encoding several Wnt antagonist chimeric constructs (Frz1-Fc (SEQ ID NO: 115), Frz2-Fc (SEQ ID NO: 116), Frz3-Fc (SEQ ID NO: 117), Frz4-Fc (SEQ ID NO: 118), Frz5-Fc (SEQ ID NO: 119), Frz6-Fc (SEQ ID NO: 120), Frz7-Fc (SEQ ID NO: 121), Frz8-Fc (SEQ ID NO: 122), Frz9-Fc (SEQ ID NO: 123), Frz10-Fc (SEQ ID NO: 124), sFRP1-Fc (SEQ ID NO: 125), sFRP2-Fc (SEQ ID NO: 126), sFRP3-Fc (SEQ ID NO: 127), sFRP4-Fc (SEQ ID NO: 128), and sFRP5-Fc (SEQ ID NO:129)).

Figure 6A-6E shows the full length amino acid sequences of the human Frz, sFRP, and Ror proteins.

Figure 7 (A, B, and C) shows the amino acid sequences of several Wnt antagonist chimeric constructs (Frz1-Fc (SEQ ID NO: 76), Frz2-Fc (SEQ ID NO: 77), Frz3-Fc (SEQ ID NO: 78), Frz4-Fc (SEQ ID NO: 79), Frz5-Fc (SEQ ID NO: 75), Frz6-Fc (SEQ ID NO: 80), Frz7-Fc (SEQ ID NO: 81), Frz8-Fc (SEQ ID NO: 74), Frz9-Fc (SEQ ID NO: 82), Frz10-Fc (SEQ ID NO: 83), sFRP1-Fc (SEQ ID NO: 84), sFRP2-Fc (SEQ ID NO: 85), sFRP3-Fc (SEQ ID NO: 86), sFRP4-Fc (SEQ ID NO: 87), and sFRP5-Fc (SEQ ID NO: 88)). The bold text for Frz1-Fc (first 28 N-terminal amino acid residues), Frz2-Fc (first 31 N-terminal amino acid residues), Frz3-Fc (first 31 N-terminal amino acid residues), Frz4-Fc (first 31 N-terminal amino acid residues) Frz5-Fc (first 31 N-terminal amino acid residues), and sFRP3-Fc (first 31 N-terminal amino acid residues) indicates a non-native leader sequence. The linker is underlined and the Fc domain, following the linker, is shown in italics.

Figure 8 shows an alignment of Frizzled extracellular domains where black shows conserved residues across all receptors and gray represents residues conserved across homologous groups.

Figure 9 shows Frizzleds grouped into families based on both full-length and extracellular domain sequence identities.

Figure 10 depicts samples of purified Frizzled-Fc fusion proteins expressed and purified from CHO cells. Samples were separated on non-reducing SDS-PAGE gels and imaged by Coomassie staining.

Figure 11 show a comparison of serum stability of the two different Frz8-Fc chimeras Frz8(173)-Fc and Frz(156)-Fc. Figure 11A is an immunoblot for human FC used to detect the chimeric proteins present at increasing time points in serum of athymic nude mice injected with the chimeras. Figure 11B shows the Wnt inhibitory activity of the chimeric proteins assayed by measuring TOPglow activity shown on the Y axis as relative luciferase activity.

Figure 12 is a graph of tumor volume over time resulting from treatment with Frz8(173)-Fc chimera.

Figure 13 shows pharmacokinetic (PK) data for Frz8-Fc following administration of a single dose of the protein. Figure 13A is an immunoblot of a neat serum from mice treated with Frz8-Fc showing detection in serum at 7 days and beyond from both 20 or 5 mg/kg I.V. or 20 mg/kg I.P. Figures 13B and 13C are a graphical summary of Frz8-Fc serum levels as determined from the pharmacokinetic study. Figure 13D is a summary of the parameters for a biphasic model of Frz8-Fc pharmacokinetics.

Figure 14 demonstrate the enhanced ability of Frz8-ECD to block Wnt3a signaling when linked to a dimeric Fc domain. Figure 14A is an IC_{50} graph of a Wnt3a inhibition assay of two different preparations of Frz8(156)-FC. Figure 14B is a gel confirming the purity of the isolated Frz8(156) CRD (ECD). Shown are: (a) non-reduced Frz8 ECD (Lane 1); (b) molecular weight markers (Lane 2); and reduced Frz8 ECD (Lane 3).

Figure 15 demonstrates direct binding of Wnt3a to the Frz8-Fc chimera. Figure 15A is BIAcore sensogram demonstrating binding of purified soluble Wnt3a to immobilized Frz8-Fc. Figure 15B is an immunoprecipitation of a purified soluble Wnt3a by immobilized Frz8-Fc.

Figure 16 demonstrates direct binding of several Frz-Fc chimeras to Wnt ligands as measured using the OCTET™ system. Figure 16A shows data from the binding of Wnt3a to the Frz1-Frz10-Fc chimeras, Figure 16B shows data from the binding of Wnt3a to sFRP-Fc chimeras. Figure 16C shows data from the binding of Wnt5a to the Frz1-Frz10-Fc chimeras and sFRP-Fc chimeras.

Figure 17 shows the effect of the Wnt antagonists on Wnt-stimulated cells transiently transfected with TOPglow luciferase TCF reporter plasmid. Figure 17A shows cells stimulated with Wnt3a and Figure 17B shows cells stimulated with Wnt-5a. Cells to be treated with Wnt5a were transfected with Frz4 and Lrp5 in addition to the reporter. 293 (human kidney) cells were activated with 100ng/ml Wnt3a or 1ug/ml Wnt5a. Cells then left

untreated, treated with control Fc, or treated with purified Frz-Fc protein in PBS and assayed for luciferase response.

Figure 18 shows inhibition of Wnt signaling by the Wnt antagonists in U2OS (human osteosarcoma) cells stably transfected with a luciferase TCF reporter plasmid. Initial Wnt signaling in cells was obtained with Wnt3a activation.

Figure 19 shows the effect of Frz8-Fc on expression of Wnt-target genes in cultured teratoma cells and tumor xenografts. Figure 19A shows expression of Wnt-target genes in PA-1 cell lines treated with Wnt3a and Frz8-Fc. RNA isolated from PA-1 cells that were treated with Wnt3a, Frz8-Fc, or control Fc protein was subject to microarray analysis and the change in expression levels of the indicated genes in response to exogenously added Wnt3a, Frz8-Fc, and control Fc protein was plotted. Columns, mean expression level from three wells; bars, standard error (S). Figure 19B shows the relative expression of Wnt target genes APCDD1, Gad-1, and Fzd5 in NTera-2 tumors from mice given PBS, CD4-Fc, or Frz8-Fc relative to PBS control. The data represents the mean expression level from the indicated number of tumors and is representative of at least two independent qRT-PCR experiments done in duplicate. Regulation of expression of each gene by the addition of purified Wnt3a to the corresponding cultured cells is also presented.

Figure 20 shows the accession number and sequence of primers and probes used for real-time quantitative PCR analysis of gene expression shown in Figure 19 (Example 9).

Figure 21 is a linear schematic describing the vector construct used in the transfection to create the Wnt animal model.

Figure 22 illustrates the efficacy of Frz8-Fc against MMTV-Wnt tumor transplants in athymic nude mice by intraperitoneal (IP) dosing. Figure 22A is a graph showing data from nude mice hosting MMTV-Wnt-1 tumor transplants were administered PBS, CD4-Fc (10 mg/kg/day) or Frz8-Fc (10 mg/kg/day) by intraperitoneal injection twice weekly. Mean tumor volume is plotted over time and the treatment days are indicated by arrows on the X-axis. Figure 22B is tabular summary of mean tumor volume and mean % change in tumor volume over time in the four treatment groups.

Figure 23 illustrates the efficacy of Frz8-Fc against MMTV-Wnt tumor transplant in athymic nude mice by intravenous (IV) dosing. Figure 23A is a graph showing data from nude mice hosting MMTV-Wnt-1 tumor transplants were administered PBS, CD4-Fc (10 mg/kg/day) or Frz8-Fc (10 mg/kg/day) by intravenous injection three times weekly. Mean tumor volume is plotted over time and the treatment days are indicated by arrows on the X-

axis. Figure 23B is a tabular summary of mean tumor volume and mean % change in tumor volume over time in the four treatment groups.

Figure 24 is a bar graph showing the Wnt signaling antagonist activity in the TOPglow assay of various Wnt antagonists in serum isolated from the MMTV Wnt tumor study. The X-axis samples appear in groups A-E (Figure 24A) or A-F (Figure 24B) according to treatment, mouse study number and dilution. The relative luciferase activity in the TOPGLOW gene reporter assay is shown on the Y-axis. All samples are treated with ~ 40 ng/ml purified Wnt3a except for NA (control). All other protein controls are present in the medium at 5 μg/ml. Figure 24A shows the testing results of serum isolated from IP treated mice, while the IV treated ones appear in Figure 24B.

Figure 25 shows Wnt signaling antagonist activity in the TOPglow assay of various Wnt antagonists in the indicated teratacarcinoma cell lines in the absence (Figure 25A) or presence (Figure 25B) of exogenously added Wnt3a. For each cell line, activity was expressed relative to that observed in the absence of any treatment (NA); representative of at least two independent experiments. Relative luciferase activity (Y-axis) were measured from TOPglow assays from various cancer cell lines in the presence or absence or Wnt inhibitors.

Figure 26 demonstrates the anti-tumor efficacy of Frz8 (156)-Fc treatment on the growth of NTera2 tumor xenografts in athymic nude mice. Figure 26A is procedural flow chart, while Figure 26B is a graph plotting mean tumor volume over time, wherein the treatment days are indicated by arrows on the X-axis. Figure 26C is a bar graph plotting the mean tumor weights at sacrifice of all animals in the group at day 20 of the study. Figures 26D and 26E are tabular summaries of mean tumor volume and mean % change in tumor volume, respectively.

Figure 27 is a bar graph showing Wnt signaling antagonist activity of serum isolated from various animals in the NTera2 tumor study as determined by the TOPglow assay. The Y-axis shows relative luciferase activity (Y-axis) from the TOPglow assay for the controls and Frz8-Fc Wnt antagonist. No additional purified Wnt or Wnt conditioned media was added to the cells.

Figure 28 shows the anti-tumor efficacy of Frz8 (156)-Fc treatment on the growth of PA-1 tumor xenografts in athymic nude mice. Figure 28A is a procedural flow chart, while Figure 28B is a graph plotting mean tumor volume over time. Figure 28C is a graph of mean tumor weight at sacrifice. The mean tumor weight ± SEM is plotted as a function of the group.

Figures 28D and 28E are tabular summaries of mean tumor volume and mean % change in tumor volume, respectively.

Figure 29 shows Wnt signaling inhibition in mice treated with Frz8-Fc or Frz5-Fz as determined by the TOPglow assay. The Y-axis shows relative luciferase activity (Y-axis) from the TOPglow assay for the controls and Frz8-Fc and Frz5-Fc Wnt antagonists.

Figure 30 shows the reduced Axin2 expression in Frz8-Fc and Frz5-Fz treated tumor with Figure 30A showing expression normalized to expression of GAPDH and Figure 30B showing expression normalized to expression of rpl19.

Figure 31 shows immunohistochemistry (IHC) photomicrographs for IHC staining of β-catenin and demonstrate that Frz8-Fc treatment on regenerative tissues such as intestine and skin appear normal. Figure 31A shows IHC for β-catenin in small intestine of PBS (A-1) control protein (A-2) and Frz8-Fc (A-3) treated mice. Figure 31B shows IHC for β-catenin in skin of PBS (B-1) control protein (B-2) and Frz8-Fc (B-3) treated mice.

Figure 32 is an illustration of active Wnt signaling in human breast cancer. Figure 32A shows Wnt-1 expression (as shown by in vitro hybridization) in normal (A-1), low grade (A-2) and high grade (A-3) human breast tumor initially reported in Wong et al., J. Pathol. 196: 145 (2002). Figure 32B shows nuclear (B-1) and cytoplasmic (B-2) localization (as shown by IHC) of β-catenin in breast cancer patients. Also shown is a Kaplan-Meier survival plot (B-3) showing patient survival probability that correlates with the indicated β-catenin expression pattern. This data was initially reported in Lin et al., P.N.A.S. (USA) 97(8): 4262-66 (2000). Figure 32C is a microarray analysis of Wnt-1 expression in a normal breast from a patient without cancer in comparison with tissue isolated from a patient with infiltrating ductal carcinoma, her-2 negative.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

A "Wnt protein" is a ligand of the Wnt signaling pathway component which binds to a Frizzled receptor so as to activate Wnt signaling. Specific examples of Wnt proteins include at least 19 members, including: Wnt-1 (RefSeq.: NM_005430), Wnt-2 (RefSeq.:NM_003391), Wnt-2B (Wnt-13) (RefSeq.: NM_004185), Wnt-3 (ReSeq. :NM_030753), Wnt3a (RefSeq.: NM_033131), Wnt-4 (RefSeq.: NM_030761), Wnt-5A (RefSeq.: NM_003392), Wnt-5B (RefSeq.: NM_032642), Wnt-6 (RefSeq.: NM_006522), Wnt-7A (RefSeq.: NM_004625), Wnt-7B (RefSeq.: NM_058238), Wnt-8A

(RefSeq.: NM_058244), Wnt-8B (RefSeq.: NM_003393), Wnt-9A (Wnt-14) (RefSeq.: NM_003395), Wnt-9B (Wnt-15) (RefSeq.: NM_003396), Wnt-10A (RefSeq.: NM_025216), Wnt-10B (RefSeq.: NM_003394), Wnt-11 (RefSeq.: NM_004626), Wnt-16 (RefSeq.: NM_016087)). While each member has varying degrees of sequence identity, each contain 23-24 conserved cysteine residues which show highly conserved spacing. McMahon, AP *et al., Trends Genet.* 8: 236-242 (1992); Miller JR., *Genome Biol.* 3(1): 3001.1-3001.15 (2002). For purposes of this invention, a Wnt protein and active variants thereof is a protein that binds to a Frizzled ECD or the CRD component of such an Frz ECD.

A "Frizzled" (Frz) protein is a Wnt signaling pathway component that is a seven-pass transmembrane receptors that binds to a Wnt protein, and further complexes with other membrane - associated Wnt signaling components, so as to transmit Wnt signaling to downstream intracellular components. Frz proteins include Frz1, Frz2, Frz3, Frz4, Frz5, Frz6, Frz7, Frz8, Frz9, and Frz10. Examples of human full length Frz proteins are hFrz1 (NP_003496) (SEQ ID NO: 1), hFrz2 (NP_001457) (SEQ ID NO: 2), hFrz3 (NP_059108) (SEQ ID NO: 3), hFrz4 (NP_036325) (SEQ ID NO: 4), hFrz5 (NP_003459) (SEQ ID NO: 5), hFrz6 (NP_003497) (SEQ ID NO: 6), hFrz7 (NP_003498) (SEQ ID NO: 7), hFrz8 (NP_114072) (SEQ ID NO: 8), hFrz9 (NP_003499) (SEQ ID NO: 9), and hFrz10 (NP_009128) (SEQ ID NO: 10) (Figures 6A -6C).

A "secreted Frizzled related protein" (sFRP) is a Wnt signaling pathway component that is a secreted extracellular polypeptide that binds to a Wnt protein. sFRP proteins include sFRP1, sFRP2, sFRP3, sFRP4, and sFRP5. Examples of human full length sFRP proteins are sFRP1 (NP_003003) (SEQ ID NO: 11), sFRP2 (NP_003004) (SEQ ID NO: 12), sFRP3 (NP_001454) (SEQ ID NO: 13), sFRP4 (NP_003005) (SEQ ID NO: 14), and sFRP5 (NP_003006) (SEQ ID NO: 15) (Figures 6C-6D).

The "Ror" protein, includes the mammalian homologs, Ror1 and Ror2, which are characterized by extracellular Frizzled-like cysteine-rich domains (CRDs) as well as membrane proximal kringle domains. Ror proteins play crucial roles in developmental morphogenesis and are associated with different components of the cytoskeleton. Ror1 co-localizes with F-actin along stress fibers, while Ror2 partially colocalizes with microtubules. Ror1 and Ror2 share about 58% overall sequence identity. Ror2 associates with the melanoma-associated antigen (MAGE) family protein Dlxin-1 and regulates its intracellular distribution. Ror1 proteins include Ror1 and Ror2. Examples of human full length Ror

proteins are hRor1 (NP_005003) (SEQ ID NO: 16), and hRor2 (NP_004551) (SEQ ID NO: 17) (Figures 6D-6E).

A "Frz domain component" is a polypeptide derived from a Frz protein, a sFRP protein, a Ror protein, or other protein, that is capable of binding with a Wnt protein. A polypeptide "derived from" a protein means a polypeptide that has an amino acid sequence that can be found within the reference protein sequence or within the sequence of active variants of the protein. Examples of a Frz domain component include a minimal cysteine rich domain (CRD) of an extracellular domain "CRD (ECD)" of a Frz protein, a sFRP protein, or a Ror protein, such as the CRD (ECD) of Frz1, Frz2, Frz3, Frz4, Frz5, Frz6, Frz7, Frz8, Frz9, Frz10, sFRP1, sFRP2, sFRP3, sFRP4, sFRP5, Ror1, or Ror2, and active variants thereof. The CRD (ECD) is a conserved structural motif of 100 to 250 amino acids and is defined by 10 highly conserved cysteines. Particular examples of human CRD (ECD)s are shown in boxed text in Figure 3B and presented as SEQ ID NOs: hFrz1 (SEQ ID NO: 18), hFrz2 (SEQ ID NO: 19), hFrz3 (SEQ ID NO: 20), hFrz4 (SEQ ID NO: 21), hFrz5 (SEQ ID NO: 22), hFrz6 (SEQ ID NO: 23), hFrz7 (SEQ ID NO: 24), hFrz8 (SEQ ID NO: 25), hFrz9 (SEQ ID NO: 26), hFrz10 (SEQ ID NO: 27), sFRP1 (SEQ ID NO: 28), sFRP2 (SEQ ID NO: 29), sFRP3 (SEQ ID NO: 30), sFRP4 (SEQ ID NO: 31), sFRP5 (SEQ ID NO: 32), hRor1 (SEQ ID NO: 33), and hRor2 (SEQ ID NO: 34).

Additional examples of a Frz domain component include a pro-Frz domain derived from a pro-Frz or pro-sFRP protein such as Frz1, Frz2, Frz3, Frz4, Frz5, Frz6, Frz7, Frz8, Frz9, Frz10, sFRP1, sFRP2, sFRP3, sFRP4, or sFRP5, and active variants thereof. Particular examples of human pro-Frz domains are shown in Figure 3A and presented as SEQ ID NOs: hFrz1 (SEQ ID NO: 35), hFrz2 (SEQ ID NO: 36), hFrz3 (SEQ ID NO: 37), hFrz4 (SEQ ID NO: 38), hFrz5 (SEQ ID NO: 39), hFrz6 (SEQ ID NO: 40), hFrz7 (SEQ ID NO: 41), hFrz8 (SEQ ID NO: 42), hFrz9 (SEQ ID NO: 43), hFrz10 (SEQ ID NO: 44), sFRP1 (SEQ ID NO: 45), sFRP2 (SEQ ID NO: 46), sFRP3 (SEQ ID NO: 47), sFRP4 (SEQ ID NO: 48), and sFRP5 (SEQ ID NO: 49).

Additional examples of a Frz domain component include a mature Frz domain derived from a mature Frz, sFRP, or Ror protein, such as Frz1, Frz2, Frz3, Frz4, Frz5, Frz6, Frz7, Frz8, Frz9, Frz10, sFRP1, sFRP2, sFRP3, sFRP4, sFRP5, Ror1, or Ror2 and active variants thereof. Particular examples of human mature Frz domains are shown in Figure 3B and presented as SEQ ID NOs: hFrz1 (SEQ ID NO: 50), hFrz2 (SEQ ID NO: 51), hFrz3 (SEQ ID NO: 52), hFrz4 (SEQ ID NO: 53), hFrz5 (SEQ ID NO: 54), hFrz6 (SEQ ID NO:

55), hFrz7 (SEQ ID NO: 56), hFrz8 (SEQ ID NO: 57), hFrz9 (SEQ ID NO: 58), hFrz10 (SEQ ID NO: 59), sFRP1 (SEQ ID NO: 60), sFRP2 (SEQ ID NO: 61), sFRP3 (SEQ ID NO: 62), sFRP4 (SEQ ID NO: 63), sFRP5 (SEQ ID NO: 64), hRor1 (SEQ ID NO: 65), and hRor2 (SEQ ID NO: 66).

A "Wnt antagonist" is a chimeric polypeptide comprising a Frz domain component and an immunoglobulin Fc domain that binds to a Wnt protein and is active by attenuating cellular Wnt signaling, or a physiological symptom resulting therefrom.

In certain embodiments, the Fc domain is a human IgG1, IgG2, IgG3 or IgG4 Fc domain. In one embodiment, the Fc domain is a human IgG1 Fc domain. Specific examples of Fc domains are shown in Figures 4, 5, and Figures 7 and in SEQ ID NO: 67 and SEQ ID NO: 68.

In some embodiments, the Frz domain component and the Fc domain are fused by a linker. The term "linker" refers to a component that tethers together the Frz domain component to the Fc domain. Linkers that are suitable for use in the invention exhibit minimal or no interference with expression, secretion and folding of the protein domains of the Wnt antagonist molecules and provide minimal or no interference with either the effector function of the Fc domain or Wnt protein interaction function of the Frz domain (*e.g.*, binding to a Wnt protein) through steric or other means. In particular embodiments, the linker is short peptide sequence. A linker sequence may also include additional amino acid residues from either the Frz domain component or Fc domain outside the minimal residues needed for activity. Preferred linkers will also provide for good serum stability and are resistant to protease cleavage. Specific examples of useful linkers appear in Figure 4, Figure 5, and Figure 7, including the sequences ESGGGGVT (SEQ ID NO: 69), LESGGGGVT (SEQ ID NO: 70), GRAQVT (SEQ ID NO: 71), WRAQVT (SEQ ID NO: 72), and ARGRAQVT (SEQ ID NO: 73). As noted above, these linkers may include additional amino acid residues from either the Frz domain component or the Fc domain outside the minimal residues needed for activity. These linkers may also comprise additional amino acid residues other than those from the Frz domain component or Fc domain component.

A "Wnt signaling pathway component" is a component that transduces a signal originating from an interaction between a Wnt protein and an Frz receptor. As the Wnt signaling pathway is complex, and involves extensive feedback regulation, there are numerous and likely not yet discovered members of the Wnt signaling pathway. Example Wnt signaling pathway components include the membrane associated proteins LRP5 and

LRP6, Axin, and Dishevelled, the extracellular Wnt interactive proteins sFRP, WIF-1, the LRP inactivating proteins Dkk and Krn, the cytoplasmic protein β-catenin, members of the β-catenin "degradation complex" APC, GSK3β, CKIα and PP2A, the nuclear transport proteins APC, pygopus and bcl9/legless, and the transcription factors TCF/LEF, Groucho and various histone acetylases such as CBP/p300 and Brg-1.

A "Wnt-mediated disorder" is a disorder, condition, or disease state characterized by aberrant Wnt signaling. In a specific aspect, the aberrant Wnt signaling is a level of Wnt signaling in a cell or tissue suspected of being diseased that exceeds the level of Wnt signaling in a similar non-diseased cell or tissue. In a specific aspect, a Wnt-mediated disorder includes cancer.

The term "cancer" refers to the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Examples of cancer include, but are not limited to: carcinoma, lymphoma, blastoma, and leukemia. More particular examples of cancers include, but are not limited to: chronic lymphocytic leukemia (CLL), lung, including non small cell (NSCLC), breast, ovarian, cervical, endometrial, prostate, colorectal, intestinal carcinoid, bladder, gastric, pancreatic, hepatic (hepatocellular), hepatoblastoma, esophageal, pulmonary adenocarcinoma, mesothelioma, synovial sarcoma, osteosarcoma, head and neck squamous cell carcinoma, juvenile nasopharyngeal angiofibromas, liposarcoma, thyroid, melanoma, basal cell carcinoma (BCC), medulloblastoma and desmoid.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not

have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

An "active" polypeptide, variant polypeptide, or fragments thereof, retain a biological activity of native or naturally-occurring component of the active polypeptide. Biological activity refers to a function mediated by the native or naturally-occurring counterpart of the active polypeptide. For example, binding or a protein-protein interaction constitutes a biological activity. In a specific sense, an active Wnt signaling pathway component is one which can effectively transduce a signal through interaction with other Wnt signaling pathway components. In another specific sense, an active Wnt antagonist is one which detectably attenuates Wnt signaling or a physiological condition resulting therefrom, relative to the level prior to administration of the Wnt antagonist.

"Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995).

"High stringency conditions", as defined herein, may be identified by those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) overnight hybridization in a solution that employs 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1%

SDS, and 10% dextran sulfate at 42°C, with a 10 minute wash at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) followed by a 10 minute high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.

"Moderately stringent conditions" may be identified as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Press, 1989, and include the use of washing solution and hybridization conditions (e.g., temperature, ionic strength and %SDS) less stringent that those described above. An example of moderately stringent conditions is overnight incubation at 37°C in a solution comprising: 20% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1 x SSC at about 37-50°C. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

The term "epitope tagged" refers to a polypeptide that is fused to a "tag polypeptide." The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the polypeptide to which it is fused. The tag polypeptide preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 and 50 amino acid residues (preferably, between about 10 and 20 amino acid residues). Example epitope tag sequences include HA, GD, c-myc, poly-His and FLAG.

"Treating" or "treatment" or "alleviation" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic disease or condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to having the disorder or those in whom the disorder is to be prevented (prophylaxis). When the Wnt-mediated disorder is cancer, a subject or mammal is successfully "treated" or shows a reduced tumor burden if, after receiving a therapeutic amount of a Wnt antagonist according to the methods of the present invention, the patient shows observable and/or measurable reduction in or absence of one or more of the following: reduction in the number of cancer cells or absence of the cancer cells; reduction in the tumor size; inhibition (i.e., slow to some extent and preferably stop) of cancer cell infiltration into peripheral organs including the spread of cancer into soft tissue and bone; inhibition (i.e., slow to some extent and preferably stop) of tumor

metastasis; inhibition, to some extent, of tumor growth; and/or relief to some extent, one or more of the symptoms associated with the specific cancer; reduced morbidity and mortality, and improvement in quality of life issues. To the extent the Wnt antagonist may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. Reduction of these signs or symptoms may also be felt by the patient.

The above parameters for assessing successful treatment and improvement in the disorder are readily measurable by routine procedures familiar to a physician. For cancer therapy, efficacy can be measured, for example, by assessing the time to disease progression (TDP) and/or determining the response rate (RR). Metastasis can be determined by staging tests and by bone scan and tests for calcium level and other enzymes to determine spread to the bone. CT scans can also be done to look for spread to the pelvis and lymph nodes in the area. Chest X-rays and measurement of liver enzyme levels by known methods are used to look for metastasis to the lungs and liver, respectively. Other routine methods for monitoring the disease include transrectal ultrasonography (TRUS) and transrectal needle biopsy (TRNB).

"Chronic" administration refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain the initial therapeutic effect (activity) for an extended period of time. "Intermittent" administration is treatment that is cyclic, or subject to periodic interruptions, as opposed to continuous or consecutive.

"Mammal" refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, *etc.* Preferably, the mammal is human.

Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

"Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating

agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN®, polyethylene glycol (PEG), and PLURONICS®.

An "effective amount" of a *Wnt* antagonist is an amount sufficient to carry out a specifically stated purpose. An "effective amount" may be determined empirically and in a routine manner, in relation to the stated purpose.

The term "therapeutically effective amount" refers to an amount of a *Wnt* antagonist effective to "treat" a Wnt-mediated disorder in a subject or mammal. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (*i.e.*, slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. See the definition herein of "treating". To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic.

A "growth inhibitory amount" of a Wnt antagonist is an amount capable of inhibiting the growth of a cell, especially tumor, *e.g.*, cancer cell, either *in vitro* or *in vivo*. A "growth inhibitory amount" of a *Wnt* antagonist for purposes of inhibiting neoplastic cell growth may be determined empirically and in a routine manner.

A "cytotoxic amount" of a Wnt antagonist is an amount capable of causing the destruction of a cell, especially tumor, *e.g.*, cancer cell, either *in vitro* or *in vivo*. A "cytotoxic amount" of a *Wnt* antagonist for purposes of inhibiting neoplastic cell growth may be determined empirically and in a routine manner.

The terms "antibody" and "immunoglobulin" are used interchangeably, and in the broadest sense, including monoclonal antibodies (*e.g.*, full length or intact monoclonal antibodies), polyclonal antibodies, multivalent antibodies, multispecific antibodies (*e.g.*, bispecific antibodies exhibiting the desired biological activity) and may also include certain antibody fragments, as described herein in greater detail. An antibody can be chimeric, human, humanized or affinity matured.

The light chain from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their

heavy chains (C_H), immunoglobulins can be assigned to different classes or isotypes. There are five classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, having heavy chains designated α, δ, ε, γ, and μ, respectively. The γ and α classes are further divided into subclasses on the basis of relatively minor differences in C_H sequence and function, *e.g.*, humans express the following subclasses: IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas *et al.*, Cellular and Molecular Biology, 4th Ed. (2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent associated of the antibody with one or more other proteins or peptides.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$ and Fv fragments, diabodies, linear antibodies (U.S.P. 5,641,870); Zapata *et al., Protein Eng.* 8(10): 1057-1062 (1995), single chain antibody molecules and multispecific antibodies formed from antibody fragments.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, and a residual "Fc" fragment, a designation reflecting the ability to crystallize readily. The Fab fragment consists of an entire light chain along with the variable region domain of the heavy chain (V_H), and the first constant domain of one heavy chain (C_H1). Each Fab fragment is monovalent with respect to antigen binding, *i.e.*, it has a single antigen-binding site. Pepsin treatment of an antibody yields a single large $F(ab')_2$ fragment which roughly corresponds to two disulfide linked Fab fragments having divalent antigen-binding activity and is still capable of cross-linking antigen. Fab' fragments differ from Fab fragments by having additional few residues at the carboxy terminus of the C_H1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The Fc fragment comprises the carboxy-terminal portions of both heavy chains held together by disulfides. The effector functions of antibodies are determined by sequences in the Fc region, which region is also the part recognized by Fc receptors (FcR) found on certain types of cells.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This fragment consists of a dimer of one heavy- and one light-chain variable region domain in tight, non-covalent association.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogenous antibodies, *i.e.* the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigen. In contract to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The term "chimeric" antibody, specifically included within the definition of monoclonal antibody, means antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences derived from another species or belonging to another antibody class or subclass, as well as fragment of such antibodies, so long as they exhibit the desired biological activity U.S.P. 4,816,567; Morrison *et al., P.N.A.S. USA* 81: 6851-6855 (1984).

"Humanized" forms of non-human (*e.g.,* rodent) antibodies are chimeric antibodies that contain minimal sequence derived from the non-human antibody. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or non-human primate having the desired antibody specificity, affinity, and capability. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human

immunoglobulin. For further details, see Jones *et al.*, *Nature* 321:522-525 (1986); Riechmann *et al.*, *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also, Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Trans.* 23: 1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5: 428-433 (1994).

"Polynucleotide" or "nucleic acid" are used interchangeably herein, and refer to polymers of nucleotides of any length, including, but are not limited to DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase, or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imported before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after synthesis, such as by conjugation with a label. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example: uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, *etc.*); charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, *etc.*); pendant moieties, such as, for example, proteins (*e.g.*, nucleases, toxins, antibodies, signal peptides, poly-L-lysine, *etc.*); intercalators (*e.g.*, acridine, psoralen, *etc.*); chelators (*e.g.*, metals, radioactive metals, boron, oxidative metal, *etc.*), alkylators, modified linkages (*e.g.*, alpha anomeric nucleic acids, *etc.*). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid or semi-solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, α-anomeric sugars, epimeric sugars such as arabinose, xylose or lyxose, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is

replace by P(O)-S-(thioate), P(S)-S-(dithioate)-, (O)NR_2-amidate, P(O)R, P(O)OR', CO or CH_2-(formacetal), in which each R or R' is independently H or substituted or unsubstituted C_{1-20} alkyl, optionally containing an ether, aryl, alkenyl, cycloalkenyl or aralkyl linkage. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

The term "peptide" generally refers to a contiguous and relatively short sequence of amino acids linked by peptidyl bonds. Typically, but not necessarily, a peptide has length of about 2 to 50 amino acids, 4-40 amino acids or 10-30 amino acids. Although the term "protein" generally refers to longer forms of a "polypeptide," the two terms can be and are used interchangeably in some contexts herein, and refer to amino acid sequences that are generally longer and perhaps more complex (*e.g.*, multiple sequence, secondary and higher structure).

A "region" of a polypeptide is a contiguous sequence of 2 or more amino acid residues. In alternative embodiments, a region is at least about 3, 5, 10, 15 or more contiguous amino acid residues.

"C-terminal region", "C-terminal sequence" and variations thereof, as used herein, refer to an amino acid sequence that is located at or in close proximity to the C-terminal (generally 3') end. Generally, the sequence includes an amino acid that has a free carboxyl group. In one embodiment, a C-terminal regions or sequence refers to a region of a polypeptide that includes about 1-15 residues located closest to the C-terminus.

"N-terminal region", "N-terminal sequence", and variations thereof, as used herein, refer to an amino acid sequence that is located at or in close proximity to the N-terminal (generally 5') end. Generally, the sequence includes an amino acid that has free amino group. In one embodiment, an N-terminal region or sequence refers to a region of a polypeptide that includes about 1-15 residues located closest to the N terminus of the polypeptide.

"Internal region" or "internal sequence", and variations thereof, refer to an amino acid sequence that is located within a polypeptide and is flanked on both its N- and C-termini by one or more amino acids that are not part of the sequence. Generally, the sequence does not include an amino acid with either a free carboxyl or amino group.

A "ligand" refers to a naturally-occurring or synthetic molecule or moiety that is capable of a binding interaction with a specific site on a protein or other molecule, such as a

receptor. A *Wnt* ligand is a molecule that specifically interacts with a *Frizzled* receptor. A "receptor" is often, but need not be located on the cell surface or membrane.

A "fusion protein" refers to a polypeptide having two portions covalently linked together, where each of the portions is derived from different proteins. The two portions may be linked directly by a single peptide bond or through a peptide linker containing one or more amino acid residues. Generally, the two portions and the linker will be in reading frame with each other and are produced using recombinant techniques.

A Wnt antagonist that "inhibits the growth of tumor cells" or a "growth inhibitory" Wnt antagonist is one which results in measurable growth inhibition of tumor cells having aberrant Wnt signaling activity. Preferred growth inhibitory Wnt antagonists inhibit growth of tumor cells having aberrant Wnt signaling activity by greater than 20%, preferably from about 20% to about 50%, and even more preferably, by greater than 50% (*e.g.*, from about 50% to about 100%) as compared to the appropriate control, the control typically being cancer cells not treated with the Wnt antagonist molecule being tested. In one embodiment, growth inhibition can be measured at a Wnt antagonist concentration of about 0.1 to 30 μg/ml or about 0.5 nM to 200 nM in cell culture, where the growth inhibition is determined 1-10 days after exposure of the tumor cells to the Wnt antagonist. Growth inhibition of tumor cells *in vivo* can be determined in various ways such as is described in the Experimental Examples section below. The Wnt antagonist is growth inhibitory *in vivo* if administration of the Wnt antagonist at about 1 μg/kg to about 100 mg/kg body weight results in reduction in tumor size or cell proliferation within about 5 days to 3 months from the first administration of the antibody, preferably within about 5 to 30 days. In a specific aspect, the tumor size is reduced relative to its size at the start of therapy.

The terms "cell proliferative disorder" and "proliferative disorder" refer to disorders that are associated with some degree of abnormal cell proliferation. In one embodiment, the cell proliferative disorder is cancer.

"Tumor", as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

A Wnt antagonist molecule which "induces cell death" is one which causes a viable cell to become nonviable. The cell is one having aberrant Wnt signaling activity as compared to a normal cell of the same tissue type. Preferably, the cell is a cancer cell, as defined herein. Cell death *in vitro* may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody-dependent cell-mediated

cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (*i.e.*, in the absence of complement) and in the absence of immune effector cells. To determine whether the *Wnt* antagonist is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore *et al.* Cytotechnology 17:1-11 (1995)) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies, oligopeptides or other organic molecules are those which induce PI uptake in the PI uptake assay in BT474 cells.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody, oligopeptide or other organic molecule so as to generate a "labeled" antibody, oligopeptide or other organic molecule. The label may be detectable by itself (*e.g.* radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.*, At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents, enzymes and fragments thereof such as nucleolytic enzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, and the various antitumor or anticancer agents disclosed below. Other cytotoxic agents are described below. A tumoricidal agent causes destruction of tumor cells.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin);

bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gamma1I and calicheamicin omegaI1 (see, *e.g.*, Agnew, Chem Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti- adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine

(ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoids, *e.g.*, TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANETM Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), and TAXOTERE® doxetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; gemcitabine (GEMZAR®); 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine (VELBAN®); platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); oxaliplatin; leucovovin; vinorelbine (NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluorometlhylornithine (DMFO); retinoids such as retinoic acid; capecitabine (XELODA®); pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATINTM) combined with 5-FU and leucovovin.

Also included in this definition are anti-hormonal agents that act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of cancer, and are often in the form of systemic, or whole-body treatment. They may be hormones themselves. Examples include anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), EVISTA® raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON® toremifene; anti-progesterones; estrogen receptor down-regulators (ERDs); agents that function to suppress or shut down the ovaries, for example, leutinizing hormone-releasing hormone (LHRH) agonists such as LUPRON® and ELIGARD® leuprolide acetate, goserelin acetate, buserelin acetate and tripterelin; other anti-androgens such as flutamide, nilutamide and bicalutamide; and aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestanie, fadrozole, RIVISOR® vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole. In addition, such definition of chemotherapeutic agents includes bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), DIDROCAL® etidronate, NE-58095, ZOMETA® zoledronic acid/zoledronate, FOSAMAX® alendronate, AREDIA® pamidronate, SKELID® tiludronate, or ACTONEL® risedronate; as well as

troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; lapatinib ditosylate (an ErbB-2 and EGFR dual tyrosine kinase small-molecule inhibitor also known as GW572016); and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially a cancer cell having *Wnt* signaling activity, either *in vitro* or *in vivo*. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of such cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topoisomerase II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami *et al.* (WB Saunders: Philadelphia, 1995), especially p. 13. The taxanes (paclitaxel and docetaxel) are anticancer drugs both derived from the yew tree. Docetaxel (TAXOTERE®, Rhone-Poulenc Rorer), derived from the European yew, is a semisynthetic analogue of paclitaxel (TAXOL®, Bristol-Myers Squibb). Paclitaxel and docetaxel promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization, which results in the inhibition of mitosis in cells.

"Doxorubicin" is an anthracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexapyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications,

usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

II. DESCRIPTION OF SPECIFIC EMBODIMENTS

The Wnt antagonists described herein are capable of binding to Wnt ligands *in vitro* and are capable of inhibiting or suppressing Wnt stimulated cell signaling. Additionally, the Wnt antagonists have a long *in vivo* half life and exhibit anti-tumor activity *in vivo*, inhibiting the growth of *Wnt*-1 driven tumors in a mouse MMTV breast tumor model. The Wnt antagonists are also capable of inhibiting the growth in mice of tumor xenografts derived from human teratoma cell lines. Regenerative tissues taken from mice that were treated with a Wnt antagonist appear to be within physiological norms. The Wnt antagonists are also capable of inhibiting autocrine Wnt signaling in human tumor cell lines *in vitro*.

The Frizzled receptor proteins can be grouped into families based on both full-length and extracellular domain sequence identities. This grouping is illustrated in the alignments shown in Figures 8 and 9. The underlined residues in this figure are conserved across all the Frz receptors and the shadowed residues are conserved across homologous groupings. The Frz proteins can be grouped into the following families 1) Frz1, Frz2, and Frz7 having a shared homology of 68-77% for the full length sequence and 90% for the ECD; 2) Frz5 and Frz8 having a shared homology of 57% for the full length sequence and 80% for the ECD; 3) Frz9 and Frz10 having a shared homology of 61% for the full length sequence and 74% for the ECD; 4) Frz3 and Frz6 having a shared homology of 49% for the full length sequence and 50% for the ECD; and 5) Frz4 (which exhibits a shared homology of 46% for the full length sequence and 48% for the ECD with Frz10). The family of Frz1, Frz2, and Frz7 also has significant homology to Drosophila Frz1 and the family of Frz5 and Frz8 has significant homology to Drosophila Frz2, shown to be responsible for planar cell polarity and Wnt signaling, respectively.

Wnt ligand-Frizzled binding behavior appears to cluster within Frizzled families. Both Wnt3a and Wnt5a bind Frz5, Frz8, and Frz4 fastest relative to the other Frz proteins while Wnt3a binds Frz1, Frz 2, and Frz7 at a slower rate. The amplitude and linear nature of Wnt5a binding behavior is indicative of lower binding affinity, relative to Wnt3a binding, as determined by the OCTET™ binding assay. The presence of both high affinity and low affinity receptors may confer ability for acute and long term signaling.

The ability of the Wnt antagonists to inhibit Wnt ligand induced signaling also appears to cluster within Frizzled families. Both Frz5 and Frz8 show complete inhibition of the Wnt3a signal and significant inhibition of the Wnt5a signal in a cell-based assay (Example 7). Frz4, Frz2, and Frz7 show significant inhibition of the Wnt3a signal. This finding mirrors the observation in Drosophila that dFrz2 (with homology to Frz 5 and Frz 8) strongly activates and dFrz1 (with homology to Frz 1, Frz2, and Frz7) can weakly activate the Wnt pathway.

While not being bound to a particular theory of action, the data presented herein indicate that cell-based Wnt signaling inhibition data generated using the Wnt antagonists correlates with data obtained by measuring the direct binding of Wnt ligands to the Wnt antagonists, indicating that the Wnt antagonists bind directly to Wnt ligands thus blocking them from binding the full-length Frizzled receptors on the cell. The data presented herein further provides validation that *in vitro* activity can be used to predict *in vivo* Wnt signaling blocking activity of the Wnt antagonists.

As indicated in the studies with Fz8-Fc set forth in the Examples, the Wnt antagonists comprising both a Frizzled domain and an immunoglobulin FC domain surprisingly exhibit increased binding affinity to Wnt ligand over the Frizzled domain alone. For example, Figure 14 shows that binding affinity increased over two orders of magnitude when the Fz ECD domain was converted to the Fz (156)-Fc construct. The finding of the Fz (156)-Fc construct as a stable and highly efficacious Wnt signaling inhibitor, in which conjugation to Fc resulted in a two order of magnitude increase in binding affinity, was greatly unexpected and non-obvious.

A. Compositions and methods of the invention

1. Polypeptides

The present invention is directed toward compositions and methods for the treatment of Wnt-mediated disorders, including cancer, and for inhibiting cellular Wnt signaling. One aspect of the invention provides Wnt antagonists that are chimeric molecules comprising a Frizzled (Frz) domain component and an immunoglobulin Fc domain. In particular embodiments of this aspect, the Frz domain component and Fc domain are fused through a linker. Another aspect provides for use of these *Wnt* antagonists for inhibiting cellular Wnt signaling and for treatment of Wnt-mediated disorders, such as cancer.

In one aspect, the invention provides for Wnt antagonists that are chimeric molecules with a Frz domain component comprising a minimal cysteine rich domain (CRD) of an extracellular domain "CRD (ECD)". The CRD (ECD) is a conserved structural motif of 100 to 250 amino acids and is defined by 10 highly conserved cysteines. This protein domain appears in two classes of the Wnt signaling family - the integral membrane Wnt receptor proteins known as Frizzled, and the secreted extracellular proteins known as the Frizzled related protein (sFrp).

In one aspect, the invention provides for Wnt antagonists that are chimeric molecules having a Frz domain component comprising a CRD (ECD) of a Frizzled protein such as Frz1, Frz2, Frz3, Frz4, Frz5, Frz6, Frz7, Frz8, Frz9, or Frz10. Examples of such CRD (ECD)s are provided in Figure 3B. In specific embodiments, the Frz domain component is selected from the group consisting of CRD (ECD)s of hFrz1 (SEQ ID NO: 18), hFrz2 (SEQ ID NO: 19), hFrz3 (SEQ ID NO: 20), hFrz4 (SEQ ID NO: 21), hFrz5 (SEQ ID NO: 22), hFrz6 (SEQ ID NO: 23), hFrz7 (SEQ ID NO: 24), hFrz8 (SEQ ID NO: 25), hFrz9 (SEQ ID NO: 26), and hFrz10 (SEQ ID NO: 27), and active variants thereof.

Alternatively, the Frz domain component comprises, for example, a CRD (ECD) from a secreted Frizzled related protein (sFRP) such as sFRP1, sFRP2, sFRP3, sFRP4, or sFRP5. Examples of such CRD (ECD)s are provided in Figure 3B. In specific embodiments, the Frz domain component is selected from the group consisting CRD (ECD)s of sFRP1 (SEQ ID NO: 28), sFRP2 (SEQ ID NO: 29), sFRP3 (SEQ ID NO: 30), sFRP4 (SEQ ID NO: 31), sFRP5 (SEQ ID NO: 32), and active variants thereof.

Alternatively, the Frz domain component comprises, for example, a CRD(ECD) of the receptor tyrosine kinases Ror1 and Ror2. Examples of such CRD (ECD)s are provided in Figure 3B. In specific embodiments, the Frz domain component is selected from the group consisting of CRD (ECD)s of hRor1 (SEQ ID NO: 33), and hRor2 (SEQ ID NO: 34), and active variants thereof.

In another aspect, the Frz domain component is a pro-Frz or pro-sFrp sequence, examples of which are shown in Figure 3A. In specific embodiments, the Frz domain component is selected from the group consisting of hFrz1 (SEQ ID NO: 35), hFrz2 (SEQ ID NO: 36), hFrz3 (SEQ ID NO: 37), hFrz4 (SEQ ID NO: 38), hFrz5 (SEQ ID NO: 39), hFrz6 (SEQ ID NO: 40), hFrz7 (SEQ ID NO: 41), hFrz8 (SEQ ID NO: 42), hFrz9 (SEQ ID NO: 43), hFrz10 (SEQ ID NO: 44), sFRP1 (SEQ ID NO: 45), sFRP2 (SEQ ID NO: 46), sFRP3

(SEQ ID NO: 47), sFRP4 (SEQ ID NO: 48), and sFRP5 (SEQ ID NO: 49), and active variants thereof.

In yet another aspect, the Frz domain component is derived from a mature Frz, sFRP or hRor sequence, examples of which are shown in Figure 3B. In specific embodiments, the Frz domain component is selected from the group consisting of hFrz1 (SEQ ID NO: 50), hFrz2 (SEQ ID NO: 51), hFrz3 (SEQ ID NO: 52), hFrz4 (SEQ ID NO: 53), hFrz5 (SEQ ID NO: 54), hFrz6 (SEQ ID NO: 55), hFrz7 (SEQ ID NO: 56), hFrz8 (SEQ ID NO: 57), hFrz9 (SEQ ID NO: 58), hFrz10 (SEQ ID NO: 59), sFRP1 (SEQ ID NO: 60), sFRP2 (SEQ ID NO: 61), sFRP3 (SEQ ID NO: 62), sFRP4 (SEQ ID NO: 63), sFRP5 (SEQ ID NO: 64), hRor1 (SEQ ID NO: 65), and hRor2 (SEQ ID NO: 66), and active variants thereof.

In particular embodiments, the Frz domain component and the immunoglobulin Fc domain of the chimeric Wnt antagonist molecules are fused through a linker. In one embodiment, the linker is a peptide linker. In another embodiment, the linker is selected from the group consisting of ESGGGGVT (SEQ ID NO: 69), LESGGGGVT (SEQ ID NO: 70), GRAQVT (SEQ ID NO: 71), WRAQVT (SEQ ID NO: 72), and ARGRAQVT (SEQ ID NO: 73). Optionally, the linkers may include additional amino acid residues from either the Frz domain component or the Fc domain outside the minimal residues needed for activity. These linkers may also comprise additional amino acid residues other than those from the Frz domain component or Fc domain component.

In one embodiment, the Wnt antagonist is Frz8-Fc chimera comprising a Frz8 CRD (ECD) and a Fc domain. In some embodiments, the Frz8-Fc chimera further comprises a linker, such as a peptide linker. In a further embodiment, the Frz8-Fc further comprises a leader sequence. In a particular embodiment, the Frz domain component comprises amino acids 1-156 of the Frz8 protein (SEQ ID NO: 8). In another embodiment, the Fc component is a human Fc. In a further embodiment, the Fc component is a human IgG Fc. In yet a further embodiment, the Frz8-Fc has a Frz domain component comprising amino acids 1-156 of the Frz8 protein fused with a linker to a human IgG Fc. In a further embodiment, the Frz8-Fc is a chimera with the amino acid sequence as shown in Figure 4B (SEQ ID NO: 74). As used in the Examples and accompanying Figures, unless otherwise noted, "Frz8-Fc" refers to the chimera shown in Figure 4B (SEQ ID NO: 74).

In a further embodiment, the Wnt antagonist is Frz5-Fc chimera comprising a Frz5 CRD (ECD) and a Fc domain. In some embodiments, the Frz5-Fc chimera further comprises a linker, such as a peptide linker. In a further embodiment, the Frz5-Fc further

comprises a leader sequence. In a particular embodiment, the Frz domain component comprises amino acids 27-155 of the Frz5 protein (SEQ ID NO: 5). In another embodiment, the Fc component is a human Fc. In a further embodiment, the Fc component is a human IgG Fc. In yet a further embodiment, the Frz5-Fc has a leader sequence and a Frz domain component comprising amino acids 27-155 of a mature Frz5 protein fused with a linker to a human IgG Fc. In a further embodiment, the Frz5-Fc is a chimera with the amino acid sequence as shown in Figure 7A (SEQ ID NO: 75). As used in the Examples and accompanying Figures, unless otherwise noted, "Frz5-Fc" refers to the chimera shown in Figure 7A (SEQ ID NO: 75).

Similarly, further embodiments include Frz1-Fc, Frz2-Fc, Frz3-Fc, Frz4-Fc, Frz6-Fc, Frz7-Fc, Frz9-Fc, Frz-10-Fc, sFRP1-Fc, sFRP2-Fc, sFRP3-Fc, sFRP4-Fc and sFRP5-Fc chimeras comprising a Frz domain component comprising a Frz CRD (ECD) from each respective Frz or sFRP protein and a Fc component. In some embodiments, the Frz-Fc chimera further comprises a linker, such as a peptide linker. In further embodiments, these chimeras comprise a leader sequence. In some embodiments, the Fc component is a human Fc. In further embodiments, the Fc component is a human IgG Fc. In yet further embodiments, these chimeras have a leader sequence and a Frz CRD (ECD) fused with a linker to a human IgG Fc. In further embodiment, these chimeras have the amino acid sequences as shown in Figures 7A, 7B and 7C (SEQ ID NOs: 76 - 88). As used in the Examples and accompanying Figures, unless otherwise noted, "Frz1-Fc, Frz2-Fc, Frz3-Fc, Frz4-Fc, Frz6-Fc, Frz7-Fc, Frz9-Fc, Frz-10-Fc, sFRP1-Fc, sFRP2-Fc, and sFRP4-Fc" refer to the respective chimeras shown in Figures 7A, 7B and 7C (SEQ ID NOs: 76 – 85, and 87).

The Wnt antagonists are stable *in vivo*. Prior constructs utilizing a Frizzled domain attached to a Fc component were rapidly degraded *in vivo* making them unsuitable for use as therapeutic compounds (Hsieh, J-C. et al., *PNAS*, 96: 3546-3551 (1999)). The Wnt antagonists described herein remain stable *in vivo* for substantially longer than the prior constructs. As shown in Example 4 (Figure 13), the Frz8-Fc Wnt antagonist displayed an *in vivo* half- life of about 4 days. Accordingly, the invention provides for Wnt antagonists that have an *in vivo* half-life of at least 1 day, 2 days, 3 days, or 4 days after being administered to a mammal.

Furthermore, as shown in Example 3 (Figure 11), the Wnt antagonists retain activity *in vivo* for substantially longer than the prior constructs. In one embodiment, the Wnt antagonist is active for at least 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6

hours, 8 hours, 10 hours, 12 hours, 14 hours, 16 hours, 18 hours, 20 hours, 22 hours, 24 hours, 30 hours, 36 hours, 40 hours, 44 hours, 48 hours, 52 hours, 56 hours, 60 hours, 64 hours, 68 hours, 72 hours, 80 hours, 90 hours, or 100 hours after being administered to a mammal. Activity is measured, for example, by testing the serum of the mammal administered the Wnt antagonist for the ability to inhibit Wnt signaling as set forth in Examples 3 and 11, or by using other methods known in the art.

2. Nucleic acids

One aspect of the invention provides for a nucleic acid encoding the Wnt antagonists described herein. In specific embodiments, the nucleic acid encodes a Wnt antagonist comprising a CRD (ECD)s of hFrz1 (SEQ ID NO: 18), hFrz2 (SEQ ID NO: 19), hFrz3 (SEQ ID NO: 20), hFrz4 (SEQ ID NO: 21), hFrz5 (SEQ ID NO: 22), hFrz6 (SEQ ID NO: 23), hFrz7 (SEQ ID NO: 24), hFrz8 (SEQ ID NO: 25), hFrz9 (SEQ ID NO: 26), hFrz10 (SEQ ID NO: 27), sFRP1 (SEQ ID NO: 28), sFRP2 (SEQ ID NO: 29), sFRP3 (SEQ ID NO: 30), sFRP4 (SEQ ID NO: 31), sFRP5 (SEQ ID NO: 32), hRor1 (SEQ ID NO: 33), or hRor2 (SEQ ID NO: 34).

In other embodiments, the nucleic acid encodes a Wnt antagonist comprising a pro-Frz or pro-sFrp proteins selected from among hFrz1 (SEQ ID NO: 35), hFrz2 (SEQ ID NO: 36), hFrz3 (SEQ ID NO: 37), hFrz4 (SEQ ID NO: 38), hFrz5 (SEQ ID NO: 39), hFrz6 (SEQ ID NO: 40), hFrz7 (SEQ ID NO: 41), hFrz8 (SEQ ID NO: 42), hFrz9 (SEQ ID NO: 43), hFrz10 (SEQ ID NO: 44), sFRP1 (SEQ ID NO: 45), sFRP2 (SEQ ID NO: 46), sFRP3 (SEQ ID NO: 47), sFRP4 (SEQ ID NO: 48), and sFRP5 (SEQ ID NO: 49).

In still other embodiments, the nucleic acid encodes a Wnt antagonist comprising a mature Frz, sFRP or hRor proteins selected from among hFrz1 (SEQ ID NO: 50), hFrz2 (SEQ ID NO: 51), hFrz3 (SEQ ID NO: 52), hFrz4 (SEQ ID NO: 53), hFrz5 (SEQ ID NO: 54), hFrz6 (SEQ ID NO: 55), hFrz7 (SEQ ID NO: 56), hFrz8 (SEQ ID NO: 57), hFrz9 (SEQ ID NO: 58), hFrz10 (SEQ ID NO: 59), sFRP1 (SEQ ID NO: 60), sFRP2 (SEQ ID NO: 61), sFRP3 (SEQ ID NO: 62), sFRP4 (SEQ ID NO: 63), sFRP5 (SEQ ID NO: 64), hRor1 (SEQ ID NO: 65), and hRor2 (SEQ ID NO: 66).

In still other embodiments, the nucleic acid encodes a Wnt antagonist comprising a Frz8-Fc (SEQ ID NO: 74), Frz5-Fc (SEQ ID NO: 75), Frz1-Fc (SEQ ID NO: 76), Frz2-Fc (SEQ ID NO: 77), Frz3-Fc (SEQ ID NO: 78), Frz4-Fc (SEQ ID NO: 79), Frz6-Fc (SEQ ID NO: 80), Frz7-Fc (SEQ ID NO: 81), Frz9-Fc (SEQ ID NO: 82), Frz10-Fc (SEQ ID NO: 83),

sFRP1-Fc (SEQ ID NO: 84), sFRP2 (SEQ ID NO: 85), sFRP3-Fc (SEQ ID NO: 86), sFRP4-Fc (SEQ ID NO: 87), or sFRP5-Fc (SEQ ID NO: 88).

In one particular embodiment, the nucleic acid encodes a Frz8-Fc and comprises the nucleic acid sequence shown in SEQ ID NO: 122 (Figure 5D). In another embodiment, the nucleic acid encodes a Frz5-Fc and comprises the nucleic acid sequence shown in SEQ ID NO: 119 (Figure 5C). In yet further embodiments, the nucleic acid encodes a Frz1-Fc, Frz2-Fc, Frz3-Fc, Frz4-Fc, Frz6-Fc, Frz7-Fc, Frz9-Fc, Frz10-Fc, sFRP1-Fc, sFRP2, sFRP3-Fc, sFRP4-Fc, or sFRP5-Fc and comprises a nucleic acid sequence shown in Figure5 (A-H). For example, the nucleic acid comprises a Frz1-Fc (SEQ ID NO: 115), Frz2-Fc (SEQ ID NO: 116), Frz3-Fc (SEQ ID NO: 117), Frz4-Fc (SEQ ID NO: 118), Frz5-Fc (SEQ ID NO: 119), Frz6-Fc (SEQ ID NO: 120), Frz7-Fc (SEQ ID NO: 121), Frz8-Fc (SEQ ID NO: 122), Frz9-Fc (SEQ ID NO: 123), Frz10-Fc (SEQ ID NO: 124), sFRP1-Fc (SEQ ID NO: 125), sFRP2-Fc (SEQ ID NO: 126), sFRP3-Fc (SEQ ID NO: 127), sFRP4-Fc (SEQ ID NO: 128), or sFRP5-Fc (SEQ ID NO:129).

Another aspect of the invention provides for nucleic acids that hybridize under high stringency conditions to the nucleic acids described above.

3. Wnt Antagonist Variants

In addition to the Wnt antagonist polypeptides described herein, it is contemplated that Wnt antagonist variants can be prepared. Such variants can be prepared by introducing appropriate nucleotide changes into the encoding DNA, and/or by synthesis of the desired variant. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the Wnt antagonist, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

A Wnt antagonist variant includes, for example, a mutation or amino acid variant in an amino acid residue in one or more domains, while still retaining biological activity. A Wnt antagonist variant also includes Wnt antagonists having at least one amino acid deletion or addition, while still retaining biological activity. The addition or deletion of the amino acid residues can particularly occur in the region surrounding the amino acid sequence where the Frz domain component and Fc domain are connected, whether or not such region contains a linker. Wnt antagonist variants have at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity with a reference Wnt antagonist polypeptide sequence. In general such variants exhibit substantially the same or greater binding affinity to a Wnt protein than the reference

sequence, *e.g.*, at least 0.75X, 0.8X, 0.9X, 1.0X, 1.25X or 1.5X, based on an art-accepted binding assay quantitation unit/metric.

In specific embodiments, the Wnt antagonist variant is a chimeric molecule comprising a Frz domain component having at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity with the CRD (ECD)s of hFrz1 (SEQ ID NO: 18), hFrz2 (SEQ ID NO: 19), hFrz3 (SEQ ID NO: 20), hFrz4 (SEQ ID NO: 21), hFrz5 (SEQ ID NO: 22), hFrz6 (SEQ ID NO: 23), hFrz7 (SEQ ID NO: 24), hFrz8 (SEQ ID NO: 25), hFrz9 (SEQ ID NO: 26), hFrz10 (SEQ ID NO: 27), sFRP1 (SEQ ID NO: 28), sFRP2 (SEQ ID NO: 29), sFRP3 (SEQ ID NO: 30), sFRP4 (SEQ ID NO: 31), sFRP5 (SEQ ID NO: 32), hRor1 (SEQ ID NO: 33), or hRor2 (SEQ ID NO: 34).

In other embodiments, the Wnt antagonist variant is a chimeric molecule comprising a Frz domain component having at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity with a pro-Frz or pro-sFrp proteins selected from among hFrz1 (SEQ ID NO: 35), hFrz2 (SEQ ID NO: 36), hFrz3 (SEQ ID NO: 37), hFrz4 (SEQ ID NO: 38), hFrz5 (SEQ ID NO: 39), hFrz6 (SEQ ID NO: 40), hFrz7 (SEQ ID NO: 41), hFrz8 (SEQ ID NO: 42), hFrz9 (SEQ ID NO: 43), hFrz10 (SEQ ID NO: 44), sFRP1 (SEQ ID NO: 45), sFRP2 (SEQ ID NO: 46), sFRP3 (SEQ ID NO: 47), sFRP4 (SEQ ID NO: 48), and sFRP5 (SEQ ID NO: 49).

In still other embodiments, the Wnt antagonist variant is a chimeric molecule comprising a Frz domain component having at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity with mature Frz, sFRP or hRor proteins selected from among hFrz1 (SEQ ID NO: 50), hFrz2 (SEQ ID NO: 51), hFrz3 (SEQ ID NO: 52), hFrz4 (SEQ ID NO: 53), hFrz5 (SEQ ID NO: 54), hFrz6 (SEQ ID NO: 55), hFrz7 (SEQ ID NO: 56), hFrz8 (SEQ ID NO: 57), hFrz9 (SEQ ID NO: 58), hFrz10 (SEQ ID NO: 59), sFRP1 (SEQ ID NO: 60), sFRP2 (SEQ ID NO: 61), sFRP3 (SEQ ID NO: 62), sFRP4 (SEQ ID NO: 63), sFRP5 (SEQ ID NO: 64), hRor1 (SEQ ID NO: 65), and hRor2 (SEQ ID NO: 66).

In still other embodiments, the Wnt antagonist variant is a chimeric molecule comprising a Frz domain component having at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity with Frz8-Fc (SEQ ID NO: 74), Frz5-Fc (SEQ ID NO: 75), Frz1-Fc (SEQ ID NO: 76), Frz2-Fc (SEQ ID NO: 77), Frz3-Fc (SEQ ID NO: 78), Frz4-Fc (SEQ ID NO: 79), Frz6-Fc (SEQ ID NO: 80), Frz7-Fc (SEQ ID NO: 81), Frz9-Fc (SEQ ID NO: 82), Frz10-Fc (SEQ

ID NO: 83), sFRP1-Fc (SEQ ID NO: 84), sFRP2 (SEQ ID NO: 85), sFRP3-Fc (SEQ ID NO: 86), sFRP4-Fc (SEQ ID NO: 87), and sFRP5-Fc (SEQ ID NO: 88).

"Percent (%) amino acid sequence identity" is defined as the percentage of amino acid residues that are identical with amino acid residues in a reference (parent) polypeptide sequence when the two sequences are aligned. To determine % amino acid identity, sequences are aligned and if necessary, gaps are introduced to achieve the maximum % sequence identity; conservative substitutions are not considered as part of the sequence identity. Amino acid sequence alignment procedures to determine percent identity are well known to those of skill in the art. Often publicly available computer software such as BLAST, BLAST2, ALIGN2 or Megalign (DNASTAR) software is used to align peptide sequences. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

When amino acid sequences are aligned, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) can be calculated as:

% amino acid sequence identity = X/Y x 100

where

X is the number of amino acid residues scored as identical matches by the sequence alignment program's or algorithm's alignment of A and B

and

Y is the total number of amino acid residues in B.

If the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

An "isolated" or "purified" peptide, polypeptide, protein or biologically active fragment is separated and/or recovered from a component of its natural environment. Contaminant components include materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous materials. Preparations having preferably less than 30%

by dry weight of non-desired contaminating material (contaminants), preferably less than 20%, 10%, and preferably less than 5% contaminants are considered to be substantially isolated. An isolated, recombinantly-produced peptide/polypeptide or biologically active portion thereof is preferably substantially free of culture medium, i.e., culture medium represents preferably less than 20%, preferably less than about 10%, and preferably less than about 5% of the volume of a peptide/polypeptide preparation. Examples of contaminants include cell debris, culture media, and substances used and produced during in vitro synthesis of the peptide/polypeptide.

Variations in the Wnt antagonist described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Patent No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the antibody or polypeptide that results in a change in the amino acid sequence as compared with the native sequence antibody or polypeptide. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains *Wnt* antagonist. Guidance in determining which amino acid residue may be inserted, substituted or deleted without adversely affecting the desired activity may be found by comparing the sequence of the *Wnt* antagonist with that of homologous known protein molecules and minimizing the number of amino acid sequence changes made in regions of high homology. Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with a serine, *i.e.*, conservative amino acid replacements. Insertions, deletions or substitutions may optionally be in the range of about 1 to 5 amino acids. The variation allowed may be determined by systematically making insertions, deletions or substitutions of amino acids in the sequence and testing the resulting variants for activity exhibited by the full-length or mature native sequence.

Wnt antagonists may be prepared by any of a number of conventional techniques. Desired peptide fragments may be chemically synthesized. An alternative approach involves generating antibody or polypeptide fragments by enzymatic digestion, *e.g.*, by treating the protein with an enzyme known to cleave proteins at sites defined by particular amino acid residues, or by digesting the DNA with suitable restriction enzymes and isolating the desired fragment. Yet another suitable technique involves isolating and amplifying a DNA fragment encoding a desired antibody or polypeptide fragment, by polymerase chain

reaction (PCR). Oligonucleotides that define the desired termini of the DNA fragment are employed at the 5' and 3' primers in the PCR.

In particular embodiments, conservative substitutions of interest are shown in Table A under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table A, or as further described below in reference to amino acid classes, are introduced and the products screened.

Table A

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr; cys	cys
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in function or immunological identity of the *Wnt* antagonist are accomplished by selecting substitutions that differ significantly in their effect

on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr; Asn; Gln

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro; and

(6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Such substituted residues also may be introduced into the conservative substitution sites or, more preferably, into the remaining (non-conserved) sites.

The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter *et al.*, Nucl. Acids Res., 13:4331 (1986); Zoller *et al.*, Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells *et al.*, Gene, 34:315 (1985)], restriction selection mutagenesis [Wells *et al.*, Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the *Wnt* antagonists of the invention.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant [Cunningham and Wells, Science, 244:1081-1085 (1989)]. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Any cysteine residue not involved in maintaining the proper conformation of the *Wnt* antagonist may also be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the *Wnt* antagonist to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.*, a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (*e.g.*, 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.*, binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the Wnt antagonist and Wnt protein. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Covalent modifications of Wnt antagonists are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of a Wnt antagonist with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C- terminal residues of the Wnt antagonist. Derivatization with bifunctional agents is useful, for instance, for crosslinking the Wnt antagonist to a water-insoluble support matrix or surface for use in the method for purifying Wnt antagonists. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as

3,3'-dithiobis(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate.

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the Wnt antagonist included within the scope of this invention comprises altering the native glycosylation pattern of the Frz, Wnt or sFRP polypeptide domains of the Wnt antagonist. "Altering the native glycosylation pattern" is defined as deleting one or more carbohydrate moieties found in native sequence of the component domains (either by removing the underlying glycosylation site or by deleting the glycosylation by chemical and/or enzymatic means), and/or adding one or more glycosylation sites that are not present in the native sequence component domain. In addition, the phrase includes qualitative changes in the glycosylation of the native proteins, involving a change in the nature and proportions of the various carbohydrate moieties present.

Glycosylation of antibodies and other polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the *Wnt* antagonist is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the sequence of the original (*i.e.*, pre-variant) Wnt antagonist. This sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA

encoding the sequence at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the Wnt antagonist is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, *e.g.*, in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Wnt antagonist may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, *et al.*, Arch. Biochem. Biophys., 259:52 (1987) and by Edge *et al.*, Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.*, Meth. Enzymol., 138:350 (1987).

Another type of covalent modification of Wnt antagonist comprises linking the sequence to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol (PEG), polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. The antibody or polypeptide also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington: The Science and Practice of Pharmacy, 20th edition, Gennaro, A., Ed., (2000).

The Wnt antagonists of the present invention may also be modified in a way to form molecules having additional chimeric nature, comprising a Wnt antagonist (i.e., Frz-, sFRP- or Ror-Fc chimera) fused to another, heterologous polypeptide or amino acid sequence.

In one embodiment, such a chimeric molecule comprises a fusion of the Wnt antagonist with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the Wnt antagonist. The presence of such epitope-tagged forms of the Wnt antagonist can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Wnt antagonist to be readily purified by affinity purification using an anti-tag

antibody or another type of affinity matrix that binds to the epitope tag. Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 [Field *et al.*, Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan *et al.*, Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky *et al.*, Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp *et al.*, BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin *et al.*, Science, 255:192-194 (1992)]; an α-tubulin epitope peptide [Skinner *et al.*, J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth *et al.*, Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)].

In alternative embodiments, the Wnt antagonists comprise a variant Fc component. For example, the Fc region may comprise a human Fc region sequence (e.g., a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (e.g, a substitution) at one or more amino acid positions including that of a hinge cysteine. In one embodiment, such variants have at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity with a reference Fc polypeptide sequence.

In one embodiment, the Fc region variant may display altered neonatal Fc receptor (FcRn) binding affinity. Such variant Fc regions may comprise an amino acid modification oat any one or more of amino acid positions 238, 252, 253, 254, 255, 256, 265, 272, 286, 288, 303, 305, 307, 309, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 386, 388, 400, 413, 415, 424, 433, 434, 435, 436, 439 or 447 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. Fc region variants with reduced binding to an FcRn may comprise an amino acid modification at any one or more of amino acid positions 252, 253, 254, 255, 288, 309, 386, 388, 400, 415, 433, 435, 436, 439 or 447 of Fc region (EU index/Kabat numbering). Alternatively, variants displaying increased binding to FcRn may comprise an amino acid modification at any one or more of amino acid positions 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434 of the Fc region (EU index/Kabat numbering).

In another embodiment, the Fc region variant may display reduced binding to an FcγR, and comprises amino acid modifications at positions 238, 239, 248, 249, 252, 254,

265, 268, 269, 270, 272, 278, 289, 292, 293, 294, 295, 296, 298, 301, 303, 322, 324, 327, 329, 333, 335, 338, 340, 373, 376, 382, 388, 389, 414, 416, 419, 434, 435, 437, 438 or 439 of the Fc region (EU index/Kabat numbering).

In yet another embodiment, the Fc region variant may display reduced binding to FcγRII and comprises amino acid modifications at any one or more of amino acid positions 238, 265, 269, 270, 292, 294, 295, 298, 303, 324, 327, 329, 333, 335, 338, 373, 376, 414, 416, 419, 435, 438 or 439 of the Fc region (EU index/Kabat numbering).

In a further embodiment, the Fc region variant may display enhanced binding to FcγRII, and comprises an amino acid modification at any one or more of amino acid positions 238, 265, 269, 270, 292, 294, 295, 298, 303, 324, 327, 329, 333, 338, 373, 376, 414, 416, 419, 435, 438 or 439 of the Fc region (EU index/Kabat numbering).

In a still further embodiment, the Fc region variant of interest may display reduced binding to an FcgRIII, and comprises an amino acid modification at one or more amino acid positions 238, 239, 249, 252, 254, 265, 268, 269, 270, 272, 278, 289, 293, 294, 295, 296, 301, 303, 322, 327, 329, 338, 340, 373, 376, 382, 388, 389, 416, 434, 435 or 437 of the Fc region (EU index/Kabat numbering).

In a still further embodiment, Fc region variants with altered (i.e, improved or diminished) C1q binding and/or complement dependent cytotoxicity (CDC) are described in WO99/51642. Such variants may comprise an amino acid substitution at one or more of amino acid positions 270, 322, 326, 327, 329, 331, 333 or 334 of the Fc region. See also, Duncan and Winter, Nature 322: 738-40 (1988); U.S.P. 5,648,260; U.S.P. 5,624,821 and WO94/29351 concerning Fc region variants.

B. Preparation of Wnt antagonists

The description below relates primarily to production of Wnt antagonist polypeptides by culturing cells transformed or transfected with a vector containing Wnt antagonist polypeptide-encoding nucleic acid. It is, of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare such *Wnt* antagonists. For instance, the appropriate amino acid sequence, or portions thereof, may be produced by direct peptide synthesis using solid-phase techniques [see, *e.g.*, Stewart *et al.*, Solid-Phase Peptide Synthesis, W.H. Freeman Co., San Francisco, CA (1969); Merrifield, J. Am. Chem. Soc., 85:2149-2154 (1963)]. *In vitro* protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be accomplished, for instance,

using an Applied Biosystems Peptide Synthesizer (Foster City, CA) using manufacturer's instructions. Various portions of the *Wnt* antagonist polypeptide may be chemically synthesized separately and combined using chemical or enzymatic methods to produce the desired sequence.

1. **Isolation of DNA Encoding Wnt Antagonist Polypeptide**

DNA encoding the sequence of the antagonists or any desired component domains of the Wnt antagonist, such as an Frz, or sFRP may be obtained from a cDNA library prepared from tissue believed to possess such sequence and to express it at a detectable level. Accordingly, a human Frz or sFRP sequence DNA can be conveniently obtained from a cDNA library prepared from human tissue. The desired DNA sequence gene may also be obtained from a genomic library or by known synthetic procedures (*e.g.*, automated nucleic acid synthesis).

Libraries can be screened with probes (such as oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Wnt antagonist polypeptide and components thereof is to use PCR methodology [Sambrook *et al.*, supra; Dieffenbach *et al.*, PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

Techniques for screening a cDNA library are well known in the art. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like ^{32}P-labeled ATP, biotinylation or enzyme labeling. Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook *et al.*, supra.

Sequences identified in such library screening methods can be compared and aligned to other known sequences deposited and available in public databases such as GenBank or other private sequence databases. Sequence identity (at either the amino acid or nucleotide level) within defined regions of the molecule or across the full-length sequence can be determined using methods known in the art and as described herein.

DNA sequence encoding Fc immunoglobulin domains may be derived from hybridoma cells secreting mAbs of the desired Fc subtype.

Nucleic acid having protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook *et al.*, supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

2. Selection and Transformation of Host Cells

Host cells are transfected or transformed with expression or cloning vectors described herein for *Wnt* antagonist polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in Mammalian Cell Biotechnology: A Practical Approach, M. Butler, ed. (IRL Press, 1991) and Sambrook *et al.*, supra.

Methods of eukaryotic cell transfection and prokaryotic cell transformation are known to the ordinarily skilled artisan, for example, $CaCl_2$, $CaPO_4$, liposome-mediated and electroporation. Depending on the host cell used, transformation is performed using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook *et al.*, supra, or electroporation is generally used for prokaryotes. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw *et al.*, Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) can be employed. General aspects of mammalian cell host system transfections have been described in U.S. Patent No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, J. Bact., 130:946 (1977) and Hsiao *et al.*, Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown *et al.*, Methods in Enzymology, 185:527-537 (1990) and Mansour *et al.*, Nature, 336:348-352 (1988).

Suitable host cells for cloning or expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *E. coli*. Various *E. coli* strains are publicly available, such as *E. coli* K12 strain MM294 (ATCC 31,446); *E. coli* X1776 (ATCC 31,537); *E. coli* strain W3110 (ATCC 27,325) and K5 772 (ATCC 53,635). Other suitable prokaryotic host cells include Enterobacteriaceae such as *Escherichia*, *e.g.*, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*, *e.g.*, *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. These examples are illustrative rather than limiting. Strain W3110 is one particularly preferred host or parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell secretes minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins endogenous to the host, with examples of such hosts including *E. coli* W3110 strain 1A2, which has the complete genotype *tonA* ; *E. coli* W3110 strain 9E4, which has the complete genotype *tonA ptr3*; *E. coli* W3110 strain 27C7 (ATCC 55,244), which has the complete genotype *tonA ptr3 phoA E15 (argF-lac)169 degP ompT kan*^{r}; *E. coli* W3110 strain 37D6, which has the complete genotype *tonA ptr3 phoA E15 (argF-lac)169 degP ompT rbs7 ilvG kan*^{r}; *E. coli* W3110 strain 40B4, which is strain 37D6 with a non-kanamycin resistant *degP* deletion mutation; and an *E. coli* strain having mutant periplasmic protease disclosed in U.S. Patent No. 4,946,783 issued 7 August 1990. Alternatively, *in vitro* methods of cloning, *e.g.*, PCR or other nucleic acid polymerase reactions, are suitable.

Full length antibody, antibody fragments, and antibody fusion proteins can be produced in bacteria, in particular when glycosylation and Fc effector function are not needed, such as when the therapeutic antibody is conjugated to a cytotoxic agent (*e.g.*, a toxin) and the immunoconjugate by itself shows effectiveness in tumor cell destruction. Full length antibodies have greater half life in circulation. Production in E. coli is faster and more cost efficient. For expression of antibody fragments and polypeptides in bacteria, see, *e.g.*, U.S. 5,648,237 (Carter et. al.), U.S. 5,789,199 (Joly *et al.*), and U.S. 5,840,523 (Simmons *et al.*) which describes translation initiation regio (TIR) and signal sequences for optimizing expression and secretion, these patents are incorporated herein by reference. After expression, the antibody is isolated from the E. coli cell paste in a soluble fraction and

can be purified through, *e.g.*, a protein A or G column depending on the isotype. Final purification can be carried out similar to the process for purifying antibody expressed e.g,, in CHO cells.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for *Wnt* antagonist polypeptide-encoding vectors. *Saccharomyces cerevisiae* is a commonly used lower eukaryotic host microorganism. Others include *Schizosaccharomyces pombe* (Beach and Nurse, Nature, 290: 140 [1981]; EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. Patent No. 4,943,529; Fleer *et al.*, Bio/Technology, 9:968-975 (1991)) such as, *e.g.*, *K. lactis* (MW98-8C, CBS683, CBS4574; Louvencourt *et al.*, J. Bacteriol., 154(2):737-742 [1983]), *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906; Van den Berg *et al.*, Bio/Technology, 8:135 (1990)), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishna *et al.*, J. Basic Microbiol., 28:265-278 [1988]); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa* (Case *et al.*, Proc. Natl. Acad. Sci. USA, 76:5259-5263 [1979]); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31 October 1990); and filamentous fungi such as, *e.g.*, *Neurospora, Penicillium, Tolypocladium* (WO 91/00357 published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, Biochem. Biophys. Res. Commun., 112:284-289 [1983]; Tilburn *et al.*, Gene, 26:205-221 [1983]; Yelton *et al.*, Proc. Natl. Acad. Sci. USA, 81: 1470-1474 [1984]) and *A. niger* (Kelly and Hynes, EMBO J., 4:475-479 [1985]). Methylotropic yeasts are suitable herein and include, but are not limited to, yeast capable of growth on methanol selected from the genera consisting of *Hansenula*, *Candida, Kloeckera, Pichia, Saccharomyces, Torulopsis,* and *Rhodotorula*. A list of specific species that are exemplary of this class of yeasts may be found in C. Anthony, The Biochemistry of Methylotrophs, 269 (1982).

Suitable host cells for the expression of glycosylated *Wnt* antagonist polypeptide are derived from multicellular organisms. Examples of invertebrate cells include insect cells such as Drosophila S2 and Spodoptera Sf9, as well as plant cells, such as cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and

the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen Virol. 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al.*, Proc. Natl. Acad. Sci. USA 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci. 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for *Wnt* antagonist polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

3. Selection and Use of a Replicable Vector

One aspect of the invention provides for the nucleic acid (*e.g.*, cDNA or genomic DNA) encoding a Wnt antagonist polypeptide inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan.

The Wnt antagonist may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Wnt antagonist polypeptide-encoding DNA that is inserted into the vector. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, *e.g.*, the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders, the latter described in U.S. Patent No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression, mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells.

Expression and cloning vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the *Wnt* antagonist -encoding nucleic acid, such as DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub *et al.*, Proc. Natl. Acad. Sci. USA, 77:4216 (1980). A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 [Stinchcomb *et al.*, Nature, 282:39 (1979); Kingsman *et al.*, Gene, 7:141 (1979); Tschemper *et al.*, Gene, 10:157 (1980)]. The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the

ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)].

Expression and cloning vectors usually contain a promoter operably linked to the *Wnt* antagonist -encoding nucleic acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems [Chang *et al.*, Nature, 275:615 (1978); Goeddel *et al.*, Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer *et al.*, Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the *Wnt* antagonist polypeptide.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman *et al.*, J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess *et al.*, J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657.

Wnt antagonist polypeptide transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

Transcription of a DNA encoding the Wnt antagonist polypeptide by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers

are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the vector at a position 5' or 3' to the *Wnt* antagonist polypeptide coding sequence, but is preferably located at a site 5' from the promoter.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding *Wnt* antagonist.

Still other methods, vectors, and host cells suitable for adaptation to the synthesis of *Wnt* antagonist polypeptide in recombinant vertebrate cell culture are described in Gething *et al.*, Nature, 293:620-625 (1981); Mantei *et al.*, Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

4. Culturing the Host Cells

One aspect of the invention provides for a host cell comprising the nucleic acid encoding the *Wnt* antagonists. The host cells used to produce the *Wnt* antagonist polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al.*, Meth. Enz. 58:44 (1979), Barnes *et al.*, Anal. Biochem.102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or

an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a *Frz*, *sFRP* or *Ror* sequence identified herein or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to the *Wnt* antagonist and encoding a specific antibody epitope.

6. Purification of Wnt Antagonist

Forms of *Wnt* antagonist polypeptide may be recovered from culture medium or from host cell lysates. If membrane-bound, it can be released from the membrane using a suitable detergent solution (*e.g.* Triton-X 100) or by enzymatic cleavage. Cells employed in expression of Wnt antagonist polypeptide can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents.

It may be desired to purify Wnt antagonist polypeptide from recombinant cell proteins or polypeptides. The following procedures are exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for

example, Sephadex G-75; protein A Sepharose columns to remove contaminants such as IgG; and metal chelating columns to bind epitope-tagged forms of the *Wnt* antagonist. Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, Methods in Enzymology, 182 (1990); Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York (1982). The purification step(s) selected will depend, for example, on the nature of the production process used and the particular *Wnt* antagonist polypeptide produced.

When using recombinant techniques, the Wnt antagonist polypeptide can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the Wnt antagonist polypeptide is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Carter *et al.*, Bio/Technology 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli.* Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the Wnt antagonist polypeptide is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The Wnt antagonist polypeptide composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2 or γ4 heavy chains (Lindmark *et al.*, J. Immunol. Meth. 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss *et al.*, EMBO J. 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the Wnt antagonist polypeptide comprises a C_H3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein

purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (*e.g.*, from about 0-0.25M salt).

C. Pharmaceutical Formulations

One aspect of the invention provides for a composition comprising a Wnt antagonist and at least one pharmaceutically acceptable carrier or excipient. Therapeutic formulations of the Wnt antagonists used in accordance with the present invention are prepared for storage by mixing the Wnt antagonists having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington: The Science and Practice of Pharmacy, 20th edition, A. Gennaro, Ed. (2000)), in the form of lyophilized formulations or aqueous solutions. A "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Additional examples of suitable carriers or diluents include, but are not limited to, water, saline, Finger's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. Except when a conventional media or agent is incompatible with an active compound, use of these compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as acetate, Tris, phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as

polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; tonicifiers such as trehalose and sodium chloride; sugars such as sucrose, mannitol, trehalose or sorbitol; surfactant such as polysorbate; salt-forming counter-ions such as sodium; metal complexes (*e.g.*, Zn-protein complexes); and/or non-ionic surfactants such as TWEEN®, PLURONICS® or polyethylene glycol (PEG). The antibody preferably comprises the antibody at a concentration of between 5-200 mg/ml, preferably between 10-100 mg/ml.

The formulations herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, in addition to a particular *Wnt* antagonist, it may be desirable to include in the one formulation, an additional antibody, *e.g.*, which binds a different epitope on the *Wnt* protein, to a different *Wnt* protein entirely, or an antibody to some other target such as a growth factor that affects the growth of the *Wnt* mediated disorder. Alternatively, or additionally, the composition may further comprise a chemotherapeutic agent, cytotoxic agent, cytokine, growth inhibitory agent, anti-hormonal agent, and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington: The Science and Practice of Pharmacy, 20th edition, A. Gennaro, Ed. (2000).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Therapeutic compositions herein generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of administration is in accord with known methods, e.g. injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial or intralesional routes, topical administration, or by sustained release systems.

Dosages and desired drug concentrations of pharmaceutical compositions of the present invention may vary depending on the particular use envisioned. The determination of the appropriate dosage or route of administration is well within the skill of an ordinary physician. Animal experiments provide reliable guidance for the determination of effective doses for human therapy. Interspecies scaling of effective doses can be performed following the principles laid down by Mordenti, J. and Chappell, W. "The use of interspecies scaling in toxicokinetics", In Toxicokinetics and New Drug Development, Yacobi et al., Eds., Pergamon Press, New York 1989, pp. 42-96.

When *in vivo* administration of a substance or molecule of the invention is employed, normal dosage amounts may vary from about 10 ng/kg to up to 100 mg/kg of mammal body weight or more per day, preferably about 1 μg/kg/day to 10 mg/kg/day, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature; see, for example, U.S. Pat. Nos. 4,657,760; 5,206,344; or 5,225,212. It is anticipated that different formulations will be effective for different treatment compounds and different disorders, that administration targeting one organ or tissue, for example, may necessitate delivery in a manner different from that to another organ or tissue.

Where sustained-release administration of a substance or molecule is desired in a formulation with release characteristics suitable for the treatment of any disease or disorder requiring administration of the substance or molecule, microencapsulation of the substance or molecule is contemplated. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsules. Microencapsulation of recombinant proteins for sustained release has been successfully performed with human growth hormone (rhGH), interferon-α,γ (rhIFN-α,–γ), interleukin-2, and MN rgp120. Johnson et al., Nat. Med., 2:795-799 (1996); Yasuda, *Biomed. Ther.*, 27:1221-1223 (1993); Hora et al., *Bio/Technology*, 8:755-758 (1990); Cleland, "Design and Production of Single Immunization Vaccines Using Polylactide Polyglycolide Microsphere Systems," in Vaccine Design: The Subunit and Adjuvant Approach, Powell and Newman, eds, (Plenum Press: New York, 1995), pp. 439-462; WO 97/03692, WO 96/40072, WO 96/07399; and U.S. Pat. No. 5,654,010.

The sustained-release formulations of these proteins may be developed using poly-lactic-coglycolic acid (PLGA) polymer due to its biocompatibility and wide range of

biodegradable properties. The degradation products of PLGA, lactic and glycolic acids, can be cleared quickly within the human body. Moreover, the degradability of this polymer can be adjusted from months to years depending on its molecular weight and composition. Lewis, "Controlled release of bioactive agents from lactide/glycolide polymer," in: M. Chasin and R. Langer (Eds.), *Biodegradable Polymers as Drug Delivery Systems* (Marcel Dekker: New York, 1990), pp. 1-41.

Additional examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT® (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

D. Methods of treating Wnt mediated disorder

The invention provides for methods of treating a Wnt-mediated disorder in a mammal suffering therefrom, comprising administering to the mammal a therapeutically effective amount of a Wnt antagonist. In one embodiment, the disorder is a cell proliferative disorder associated with aberrant, e.g., increased, expression of activity of Wnt signaling. In another embodiment, the disorder results from increased expression of a Wnt protein. In yet another embodiment, the cell proliferative disorder is cancer, such as for example, colon cancer, colorectal cancer, breast cancer, cancer associated with various disorders relating to HSC's, such as leukemias and various other blood related cancers, and cancer related to neuronal proliferative disorders, including brain tumors, such as gliomas, astrocytomas, meningiomas, Schwannomas, pituitary tumors, primitive neuroectodermal tumors (PNET), medulloblastomas, craniopharyngioma, and pineal region tumors.

Treatment of the cell proliferative disorder by administration of a Wnt antagonist results in an observable and/or measurable reduction in or absence of one or more of the following: reduction in the number of cancer cells or absence of the cancer cells; reduction in the tumor size; inhibition (i.e., slow to some extent and preferably stop) of cancer cell infiltration into peripheral organs including the spread of cancer into soft tissue and bone; inhibition (i.e., slow to some extent and preferably stop) of tumor metastasis; inhibition, to some extent, of tumor growth; and/or relief to some extent, one or more of the symptoms associated with the specific cancer; reduced morbidity and mortality, and improvement in quality of life issues. To the extent the Wnt antagonist may prevent growth and/or kill

existing cancer cells, it may be cytostatic and/or cytotoxic. Reduction of these signs or symptoms may also be felt by the patient.

The above parameters for assessing successful treatment and improvement in the disease are readily measurable by routine procedures familiar to a physician. For cancer therapy, efficacy can be measured, for example, by assessing the time to disease progression (TDP) and/or determining the response rate (RR). Metastasis can be determined by staging tests and by bone scan and tests for calcium level and other enzymes to determine spread to the bone. CT scans can also be done to look for spread to the pelvis and lymph nodes in the area. Chest X-rays and measurement of liver enzyme levels by known methods are used to look for metastasis to the lungs and liver, respectively. Other routine methods for monitoring the disease include transrectal ultrasonography (TRUS) and transrectal needle biopsy (TRNB).

In a specific embodiment, the administration of Wnt antagonist decreases tumor burden (e.g., reduces size or severity of the cancer). In yet another specific embodiment, the administration of Wnt antagonist kills the cancer.

E. Methods of inhibiting Wnt-signaling in a cell

The invention provides for a method of inhibiting Wnt-signaling in a cell comprising contacting the cell with an effective amount of a Wnt antagonist. In one embodiment, the cell is contained within a mammal, preferably a human, and the administered amount is a therapeutically effective amount. In yet another embodiment, the inhibition of Wnt signaling further results in the inhibition of the growth of the cell. In a further embodiment, the cell is a cancer cell.

Inhibition of cell proliferation is measured using methods known to those skilled in the art. For example, a convenient assay for measuring cell proliferation is the CellTiter-Glo™ Luminescent Cell Viability Assay, which is commercially available from Promega (Madison, WI). That assay determines the number of viable cells in culture based on quantitation of ATP present, which is an indication of metabolically active cells. *See* Crouch et al (1993) *J. Immunol. Meth.* 160:81-88, US Pat. No. 6602677. The assay may be conducted in 96- or 384-well format, making it amenable to automated high-throughput screening (HTS). *See* Cree et al (1995) *AntiCancer Drugs* 6:398-404. The assay procedure involves adding a single reagent (CellTiter-Glo® Reagent) directly to cultured cells. This results in cell lysis and generation of a luminescent signal produced by a luciferase reaction. The luminescent signal is proportional to the amount of ATP present, which is directly

proportional to the number of viable cells present in culture. Data can be recorded by luminometer or CCD camera imaging device. The luminescence output is expressed as relative light units (RLU).

F. Methods of modulating the expression of a Wnt target gene

The invention provides for a method of modulating the expression of a Wnt target gene in a cell characterized by activated or excessive Wnt signaling, comprising contacting the cell with an effective amount of a Wnt antagonist. In one embodiment, the Wnt target gene is overexpressed as a result of the Wnt signaling, and the result of the contact with the Wnt antagonist reduces expression of the Wnt target gene. In another embodiment, the Wnt target gene is selected from the group consisting of: Axin2, APCDD1, Gad1, Sax1, c-myc, cyclin D1, PPARdelta, gastrin, clusterin, survivin, cyclooxygenase, fra-1, osteopontin, uPAR, claudin-1, CD44, MMP-7/9/11/14/26, IGFBP-4, Met, BMP4, sox-9, histone deacetylase 2, VEGF. In yet another embodiment, the Wnt target gene is underexpressed as a result of the Wnt signaling, and the result of contact with the Wnt antagonist restores expression of the Wnt target gene. In a further embodiment, the Wnt target gene is selected from the group consisting of: Lefty1, Lefty2, sFRP1, Fzd5, fas antigen, caspase 3, integrin β7, alpha e integrin, hath 1, fatty acid binding protein 2, muc-2, kruppel like factor-4, carbonic anhydrase-11, EphrinB1, EphB2R, EphB3R, muc-3, histocompatibility 2, Q region locus 1, β2-microglobulin.

Expression of the target genes is determined using methods known to those of skill in the art, including those described herein and set forth in the Examples below.

G. Methods of detecting the presence of a Wnt protein

The invention provides for a method of detecting the presence of a Wnt protein in a sample, comprising contacting the sample with a Wnt antagonist, wherein the presence of a complex or the level of binding between the Wnt antagonist and the Wnt protein is indicative of the presence of Wnt protein and/or Wnt signaling. In one embodiment, the method further comprises determining if the level of Wnt signaling is aberrant. In this embodiment, the level of Wnt protein binding in the sample is compared with the level in a second sample in which Wnt protein expression and/or Wnt signaling is known to be physiologically normal. The level of binding in the suspect sample compared to the second sample that is higher or lower than the physiologically normal sample is indicative of aberrant Wnt signaling. In another embodiment, the presence of Wnt signaling or aberrant Wnt signaling is indicative of the presence of a Wnt-mediated disorder, such as cancer.

H. The Wnt pathway and disorders associated therewith

1. The Wnt signaling pathway:

The Wnt signaling pathway is an unusually complex signaling process involving multiple proteins which exert varying levels of control in the pathway. This multi-level, tight regulation of the pathway is indicative of its importance in cellular biology. Despite the complicated regulatory mechanisms, the initial signal of the pathway is generated by the binding of a Wnt to the Frizzled (Frz) receptors. Effective signal further requires the presence of an additional single pass transmembrane molecule of the LRP (LDL receptor related protein) class, specifically LRP 5 and LRP 6. Wnt may further bind with LRP to form a trimeric complex with Frizzled. The cytoplasmic tail of LRP in turn interacts with Axin, another downstream component. Dishevelled, a cytoplasmic component that interacts directly with Frizzled, may also directly interact with Axin, thus forming a tetra-plex complex of Frizzled, LRP, Dsh and Axin. This interaction with Axin releases β-catenin from the "degradation complex" (discussed infra) for subsequent downstream activity in the Wnt signaling pathway.

Outside the cell, *Wnt* signaling is inhibited by various proteins that can bind to *Wnt* thereby sequestering it from its receptor. Included in this group are the secreted *Frizzled* related proteins (*sFRPs*, Jones *et al.*, *Bioessays* 2002; **24**: 811-820) and *Wnt inhibitory factor-1* (*WIF-1*, Hsieh, J.C. *et al., Nature* 1999; **398**: 431-436). In humans, the *sFRP* family consists of five members (*e.g.*, *sFRP-1*, *sFRP-2* . . . *sFRP-5*), each containing a cysteine-rich domain (CRD) which shares 30-50% sequence homology with the CRD of *Frz* receptors. (Melkonyan, H.S. *et al., Proc. Natl. Acad. Sci. USA* 1997; **94**: 13636-13641). sFRPs are believed to form function-inhibiting complexes with *Frz* receptors, and therefore are natural antagonists, but the biology is complex, and in some cases, may even act to agonize *Wnt* activity. (Uren, A. *et al., J. Biol. Chem.* 2000; **275**: 4374-4382).

Another class of extracellular Wnt inhibitor is Dickkopf (Dkk). [Brott, B.K. *et al., Mol. Cell Biol.* 2002; **22**: 6100-6110; Fedi, P. *et al., J. Biol. Chem.* 1999; **274**: 19465-19472] The three members of the Dkk family (e.g., Dkk-1, Dkk-2 and Dkk-4) can antagonize Wnt signaling through inactivation of the cell surface receptor LRP-5 and LRP-6, essential components of the canonical pathway. [Mao, J.H. *et al., Mol. Cell* 2001; **7**: 801-809; Pinson, K.I. *et al., Nature* 2000; **407**: 535-538]. Dkk forms a ternary complex with LRP5/6 and the single pass transmembrane receptors Kremen 1 (Krm-1) or Kremen 2 (Krm-2) [Mao *et al., Gene* 2003; **302**: 179-183; Mao *et al., Nature* 2002; **417**: 664-667; Mao *et al., Nature* 2001;

411: 321-325]. This complex in turn undergoes endocytosis, thereby removing LRP5/6 receptors from the cell surface. As a result, Dkks can selectively antagonize canonical Wnt signaling, while not affecting non-canonical signaling.

The hallmark of canonical Wnt signaling activation is elevated levels of the protein β-catenin. β-catenin is constitutively produced and is present in the cytoplasm as pools of monomeric protein. [Papkoff, J. *et al., Mol. Cell Biol.* 1996; **16**: 2128-2134]. The primary mechanism for controlling cytoplasmic levels of β–*catenin* is through direct physical degradation upon recruitment into a large multi-protein complex ("degradation complex"). The central scaffolding of this complex is provided by Axin, as well as binding sites for β-catenin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), casein kinase Iα (CKIα) and protein phophatase 2A (PP2A) [Hinoi, T. et al., J. *Biol. Chem.* 2000; **275**: 34399-34406; Ikeda *et al., Oncogene* 2000; **19**: 537-545; Yamamoto *et al., J. Biol. Chem.* 1999; **274**: 10681-10684; Kishida *et al., J. Biol. Chem.* 1998; **273**: 10823-10826; Ikeda *et al., EMBO J.* 1998; **17**: 1371-1384. After formation, the complex is stabilized by the *GSK3β*-mediated phosphorylation of Axin and APC, as well as PP2A. GSK3β- then phosphorylates β-catenin thereby allowing it be recognized by β-transducin repeat containing protein (β-TrCP), thereby targeting it for ubiquitination and proteosomic degradation. [Aberle *et al., EMBO J.* 1997; **16**: 3797-804; Latres *et al., Oncogene* 1999; **18**: 849-54; Liu *et al., Proc. Natl. Acad. Sci. USA* 1999; **96**: 6273-8].

Although complexation with Axin/APC/GSK3β is the primary mechanism for degradation of β–catenin, an alternative degradation pathway has been shown involving ubiquitination induced by complexation with Siah-1 and the C-terminus of APC. [Matsuzawa *et al., Mol. Cell* 2001; **7**: 915-926; Liu *et al., Mol. Cell* 2001; **7**: 927-936]. In addition to its role as a transcription factor, β-*catenin* further is involved in cellular adhesion. [Nelson *et al., Science* 2004; **303**: 1483-1487; Ilyas *et al., J. Pathol.* 1997; **182**: 128-137. β-catenin can be found at the cell surface sites of intercellular contact known as adherens junctions, where it is complexed with E-cadherin and α-catenin. Thus, any increase in E-cadherin expression will direct β-catenin to the cell membrane, thereby depleting cytoplasmic levels, and in turn inhibit Wnt signaling. Moreover, the breakdown of the E-cadherin-catenin complex can increase cytoplasmic levels of free β-catenin, thereby stimulating transcriptional activity. [Nelson *et al., supra.*]. Thus, activation of the cell surface receptors cRON, epidermal growth factor receptor (EGFR) and c-ErbB2, by liberating β-catenin, can also stimulate canonical Wnt signaling. Other signaling pathways

that can either activate or facilitate the effects of Wnt signaling. For example, integrin signaling can result in nuclear transportation of β-catenin [Eger *et al., Oncogene* 2004; **23**: 2672-2680], while signaling through insulin-like growth factor (IGF) can activate Wnt signaling by "soaking up" available GSK3β - thereby preventing formation of the "degradation complex."

In canonical signaling, an initial step involves the binding of Wnt to Frz in the presence of LRP5/6. [Mao *et al., Mol. Cell* 2001; **7**: 801-809; Pinson *et al., Nature* 2000; **407**: 535-538]. The formation of this trimeric complex has two downstream consequences. First is the recruitment of Dishevelled (Dsh) to the cell surface and its phosphorylation by casein kinase Iε (CIε) [Kishida *et al., J. Biol. Chem.* 2001; **276**: 33147-33155]. The phosphorylated Dsh can form a complex with Frat 1 and GSK3β, which in turn can inhibit the activity of GSK3β. Second, the Wnt/Frz/LRP5/6 tri-plex facilitates the LRP5/6 mediated degradation of Axin. The net effect of this is the destabilization of the degradation complex responsible for phosphorylating β-catenin. In the absence of phosphorylation, β-catenin is not ubiquinated, thereby escaping degradation, thus increasing intracellular levels and availability for translocation to the nucleus.

The manner in which β-catenin is transported to the nucleus is not entirely clear, but interaction with the nuclear transport proteins APC [Rosin-Arbesfeld *et al., Nature* 2000; **406**: 1009-1012; Neufeld *et al., Proc. Natl. Acad. Sci. USA* 2000; **97**: 12085-12090], as well as *pygopus* and *Bcl9/legless* have been implicated. [Townsley *et al., Nature Cell Biol.* 2004; **6**: 626-633].

Once in the nucleus, β-catenin displaces the transcriptional repressor Groucho for binding with T-cell-specific transcription factor/lymphoid enhancer-binding factor-1 (TCF/LEF) DNA binding proteins. In the absence of displacement by β-catenin, TCF/LEF complexes with Groucho to repress expression of the Wnt "target genes". The inhibitory effect of *Groucho* is further mediated by interactions with various histone deacetylases (HDAC), which are believed to make DNA refractive to transcriptional activation. [Cavallo *et al., Nature* 1998; **395**: 604-8; Chen *et al., Genes Dev.* 1999; **13**: 2218-30]. The conversion of the *TCF* transcriptional repressor complex into a transcriptional activation complex further involves recruitment of histone acetylases such as Creb binding protein (CBP)/p300 as well as other activating factors such as Brg-1. [Takemaru *et al., J. Cell Biol.* 2000; **149**: 249-54; Barker *et al., Cell* 2002; **109**: 47-60; Brantjes *et al., Biol. Chem.* 2002; **383**: 255-261; Roose *et al., Biochim. Biophys Acta - Rev. Cancer* 1999; **1424**: M23-M37].

The interactions between the β-*catenin-TCF* complex and chromatin also may be mediated by *Legless (Bcl9)* and *Pygopus*. Kramps *et al., Cell* 2002; **109**: 47-60; Thompson *et al., Nat. Cell Biol.* 2002; **4:** 367-73; Parker *et al., Development* 2002; **129**: 2565-76.

An abbreviated summary of the canonical *Wnt* signaling pathway both in the "off" or inactive state as well as the "on" or active state is depicted in Figure 1.

2. Disorders associated with *Wnt* signaling activity:

Deregulation of the Wnt signaling pathway may be caused by somatic mutations in genes encoding various Wnt signaling pathway components. For example, aberrant Wnt signaling activity has been associated with Wnt ligand overexpression in non small cell lung cancer (NSCLC) [You *et al., Oncogene* 2004; **23**: 6170-6174], chronic lymphocytic leukemia (CLL)[Lu *et al., Proc. Natl. Acad. Sci. USA* 2004; **101**: 3118-3123], gastric cancer [Kim *et al., Exp. Oncol.* 2003; **25**: 211-215; Saitoh *et al., Int. J. Mol. Med.* 2002; **9:** 515-519], head and neck squamous cell carcinoma (HNSCC) [Rhee *et al., Oncogene* 2002; **21**: 6598-6605], colorectal cancer [Holcombe *et al., J. Clin. Pathol - Mol. Pathol.* 2002; **55**: 220-226], ovarian cancer [Ricken et al., Endocrinology 2002; 143: 2741-2749], basal cell carcinoma (BCC) [Lo Muzio *et al., Anticancer Res.* 2002; **22**: 565-576] and breast cancer. Moreover, the reduction of various *Wnt* ligand regulatory molecules such as *sFRP* and *WIF-1* have been associated with breast cancer [Klopocki *et al., Int. J. Oncol.* 2004; **25**: 641-649; Ugolini *et al., Oncogene* 2001; **20**: 5810-5817; Wissmann *et al., J. Pathol.* 2003; **201**: 204-212], bladder cancer [Stoehr *et al., Lab Invest.* 2004; **84**: 465-478; Wissmann *et al., supra*], mesothelioma [Lee *et al., Oncogene* 2004; **23**: 6672-6676], colorectal cancer [Suzuki *et al., Nature Genet* 2004; **36**: 417-422; Kim *et al., Mol. Cancer Ther.* 2002; **1:** 1355-1359; Caldwell *et al., Cancer Res.* 2004; **64**: 883-888], prostate cancer [Wissman *et al., supra*], NSCLC [Mazieres *et al., Cancer Res.* 2004; **64**: 4717-4720], and lung cancer [Wissman *et al., supra*]. Antagonizing *Wnt* signaling with the *Wnt* antagonist molecules of the invention is expected to therapeutically treat these cancers.

Continuing, aberrant *Wnt* signaling resulting from overexpression of various components of the Frz-LRP receptor complex have also been associated with certain cancers. For example, LRP5 overexpression has been associated with osteosarcoma [Hoang *et al., Int. J. Cancer* 2004; **109**: 106-111], while *Frz* overexpression has been associated with cancers such as prostate [Wissmann *et al., supra*], HNSCC [Rhee *et al., Oncogene* 2002; **21**: 6598-6605], colorectal [Holcombe *et al., supra*], ovarian cancer [Wissman *et al, supra*], esophageal [Tanaka *et al., Proc. Natl. Acad. Sci. USA* 1998; **95**: 10164-10169] and gastric

[Kirikoshi *et al., Int. J. Oncol.* 2001; **19**: 111-115]. Additionally, overexpression of Wnt signaling pathway components such as *Dishevelled* have been associated with cancers such as prostate [Wissman *et al., supra*], breast [Nagahata *et al., Cancer Sci.* 2003; **94**: 515-518], mesothelioma [Uematsu *et al., Cancer Res.* 2003; **63**: 4547-4551] and cervical [Okino *et al., Oncol. Rep.* 2003; **10**: 1219-1223]. Frat-1 overexpression has been associated with cancers such as pancreatic, esophageal, cervical, breast and gastric. [Saitoh *et al., Int. J. Oncol.* 2002; **20**: 785-789; Saitoh *et al., Int.J. Oncol.* 2001; **19**: 311-315]. Axin loss of function (LOF) mutations have been associated with hepatocellular cancer [Satoh *et al., Nature Genet.* 2000; **24**: 245-250; Taniguchi *et al., Oncogene* 2002; **21**: 4863-4871] and medulloblastoma [Dahmen *et al., Cancer Res.* 2001; **61**: 7039-7043; Yokota *et al., Int. J. Cancer* 2002; **101**: 198-201]. The blocking of Wnt-Frz interactions with the Wnt antagonists of the present invention is expected to alleviate cancers associated with overexpression of Frz or LRPs.

Finally, a multitude of cancers has been associated with activating β-catenin through disruption of the "degradation complex" such as gain-of-function mutations in β–catenin or loss-of-function mutations in APC. A reduction in the degradation of β-catenin results in greater amounts of functional β-catenin in the cell, which then causes increased transcription of the target genes, resulting in aberrant cell proliferation. For example, mutations in the gene encoding β-catenin (*i.e.*, CTNNB1) have been associated with cancers such as gastric [Clements *et al., Cancer Res.* 2002; **62**: 3503-3506; Park *et al., Cancer Res.* 1999; **59**: 4257-4260], colorectal [Morin *et al., Science* 1997; **275**: 1787-1790; Ilyas *et al., Proc. Natl Acad. Sci. USA* 1997; **94**: 10330-10334], intestinal carcinoid [Fujimori *et al., Cancer Res.* 2001; **61**: 6656-6659], ovarian [Sunaga *et al., Genes Chrom. Cancer* 2001; **30**: 316-321], pulmonary adenocarcinoma [Sunaga *et al., supra*], endometrial [Fukuchi *et al., Cancer Res.* 1998; **58**: 3526-3528; Kobayashi *et al., Japan. J. Cancer Res.* 1999; **90**: 55-59; Mirabelli-Primdahl *et al., Cancer Res.* 1999; **59**: 3346-3351], hepatocellular [*Satoh et al., supra.*; Wong *et al., Cancer* 2001; **92**: 136-145], hepatoblastoma [Koch *et al., Cancer Res.* 1999; **59**: 269-273], medulloblastoma [Koch *et al., Int. J. Cancer* 2001; **93**: 445-449], pancreatic [Abraham *et al., Am. J. Pathol.* 2002; **160**: 1361-1369], thyroid [Garcia-Rostan *et al., Cancer Res.* 1999; **59**: 1811-1815; Garcia-Rostan *et al., Am. J. Pathol.* 2001; **158**: 987-996], prostate [Chesire *et al., Prostate* 2000; **45**: 323-334; Voeller *et al., Cancer Res.* 1998; **58**: 2520-2523], melanoma [Reifenberger *et al., Int. J. Cancer* 2002; **100**: 549-556], pilomatricoma [Chan *et al., Nature Genet.* 1999; **21**: 410-413], Wilms' tumor [Koesters *et al., J. Pathol.* 2003; **199**: 68-76], pancreatoblastomas [Abraham *et al., Am. J. Pathol.* 2001; **159**: 1619-1627], liposarcomas [Sakamoto *et al., Arch. Pathol. Lab Med.* 2002; **126**: 1071-

1078], juvenile nasopharyngeal angiofibromas [Abraham *et al., Am. J. Pathol.* 2001; **158**: 1073-1078], desmoid [Tejpar *et al., Oncogene* 1999; **18**: 6615-6620; Miyoshi *et al., Oncol. Res.* 1998; **10**: 591-594], synovial sarcoma [Saito *et al., J. Pathol.* 2000; **192**: 342-350]. While loss-of-function mutations have been associated with cancers such as colorectal [Fearon *et al., Cell* 1990; **61**: 759-767; Rowan *et al., Proc. Natl Acad. Sci. USA* 2000; **97**: 3352-3357], melanoma [Reifenberger *et al., Int. J. Cancer* 2002; **100**: 549-556; Rubinfeld *et al., Science* 1997; **275**: 1790-1792], medulloblastoma [Koch *et al., Int. J. Cancer* 2001; **93**: 445-449; Huang *et al., Am. J. Pathol.* 2000; **156**: 433-437] and desmoids [Tejpar *et al., Oncogene* 1999; **18**: 6615-6620; Alman *et al., Am J. Pathol.* 1997; **151**: 329-334]. Cancers that result from aberrant activity of β-catenin thereby activating the Wnt pathway are suitable for treatment with the Wnt antagonists of the present invention.

3. Wnt signaling and carcinogenesis

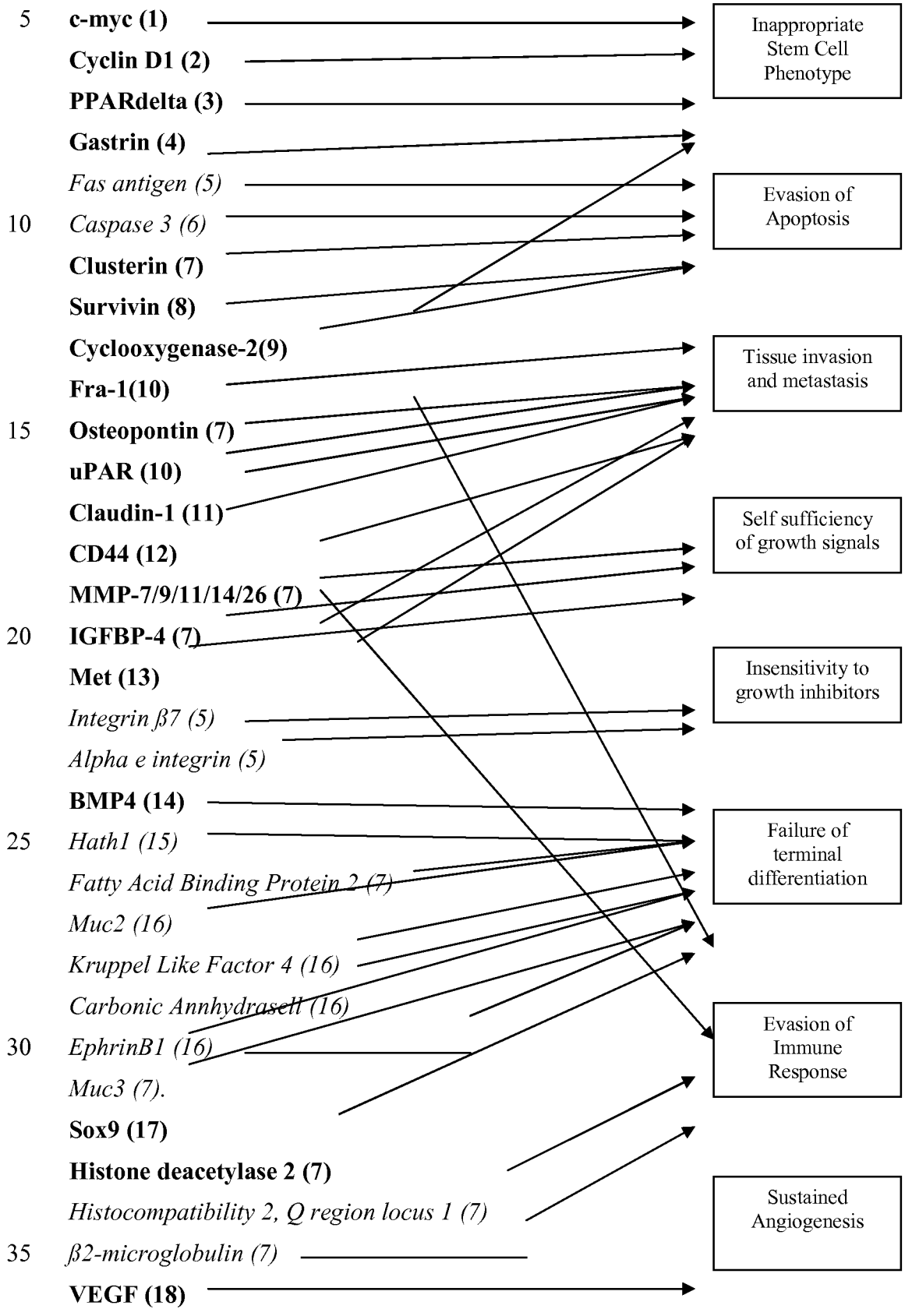
The Wnt pathway has many transcriptional endpoints or target genes. The majority of these are specific to certain types - which is not unusual in developmental signaling pathways. This is consistent with a fundamental mechanism of gene control by extracellular signals in which the cell rather than the signal determines the nature of the response. However, in addition to cell type specific genes, Wnt signaling also controls genes that are more widely induced, including components of the Wnt signaling pathway and genes that are most likely activated by the Wnt-β-catenin-TCF cascade.

The transition of normal cellular physiology into one characterized by neoplastic change has been the object of intense study in an effort to better understand the events underlying the development of cancer. The inappropriate activation of the target genes by β-*catenin* thus can result in a disease state in the organism even though there may not be any somatic mutation in the target genes themselves. Ilyas has generated a modification of the Hanahan and Weinberg list of phenotypes that are acquired by most malignancies; including "Inappropriate stem cell phenotype/limitless replicative potential", "evasion of apoptosis," "tissue invasion and metastasis," "self sufficiency of growth signals," "insensitivity to growth inhibitors," "failure of terminal differentiation," "evasion of immune response," and "sustained angiogenesis." Ilyas, *J. Pathol.* 2005; **205**: 130-144; Hanahan and Weinberg, *Cell* 2000; **100**: 57-70. Analysis of the genes modulated by *Wnt* signaling, including target genes of β-*catenin* or altered expression as shown by microarray analysis shows that the perturbations from aberrant *Wnt* signaling either directly or through the effect on target genes can impart nearly all of these "neoplastic phenotypes." Ilyas, M., *J. Pathol.* 2005;

205: 130-144. A list of example targets of *Wnt* signaling is given in Table 1. Gene targets that are upregulated appear in boldface, while those which are downregulated are italicized. Aberrant expression of such target genes due to the result of activated and/or excessive Wnt signaling may be remedies upon application of the *Wnt* antagonists of the invention.

Table 1

***Wnt* target genes and effects phenotypic effects**



Increasingly, cancer is being viewed as a "stem cell" disease (Taipale *et al., Nature* 2001; **411**: 349-54 - that is, an inappropriate activation and/or maintenance of stem cells. *Wnt* signaling has been shown to be essential for the maintenance of stem cells [He *et al., Nature Genet.* 2004; **36**: 1117-1121; Reya *et al., Nature* 2003; **423**: 409-414; Willert *et al., Nature* 2003; **423:** 448-452]. In the intestine, *TCF4* is the main nuclear binding factor for β-catenin and the failure of TCF4 knock out mice to develop stem cells in the small intestine further supports the role of canonical *Wnt* signaling in stem cell maintenance [Korinek *et al., Nat. Genet.* 1998; **19**: 379-83; Pinto *et al., Genes Dev.* 2003; **17**: 1709-13; Kuhnert *et al., Proc. Natl. Acad. Sc. USA* 2004; **101**: 66-71].

The effect of Wnt signaling on multiple biological processes is illustrated by the matrix metalloproteinase genes (MMPs). MMP7, MMP14 and MMP26 have been shown to direct targets of β-*catenin* [Marchenko *et al., Int. J. Biochem. Cell Biol.* 2004; 36: 942-956; Takahashi *et al., Oncogene* 2002; 21: 5861-5867; Brabletz *et al., Am. J. Pathol.* 1999; **155**: 1033-1038], while other MMPs were found expressed directly by intestinal adenomas [Paoni *et al., Physiol. Genomics* 2003; **15**: 228-235]. The MMPs are proteolytic enzymes that breakdown stromal collagen thereby allowing tumor cells to acquire the phenotype "tissue invasion and metastasis." The enzymatic activity also allows the release of latent growth factors in the stroma, which together with other growth factors secreted by the tumor cells themselves will contribute to "self sufficiency of growth signals." [Coussens *et al., Science* 2002; **295**: 2387-2392; Egeblad *et al., Nature Rev. Cancer* 2002; **2:** 161-174]. MMPs can also act on osteopontin (a secondary Wnt-induced target [Paoni *et al., supra*], to release fragments which together with vascular endothelial growth factor (VEGF), a direct target of β-catenin, contributes to the feature of "sustained angiogenesis." [Zhang *et al., Cancer Res.* 2001; **61**: 6050-6054; Agnihotri *et al., J. Bio. Chem.* 2001; **276**: 28261-28267].

While the Wnt signaling pathway can be activated at levels downstream of the ligand receptor interaction, there is strong evidence to suggest inhibition of the extracellular ligand-receptor interaction component is effective in reducing the tumorigenicity, even though the event initiating the *Wnt* signaling may have occurred downstream. For example, Ilyas reports in a recent review that the inhibition of *Wnt* signals in several colorectal cancer cell lines results in reduced tumorigenicity. [Ilyas, *supra.*]. Moreover, the transfection of inoperative frizzled receptor (Frz7 ectodomain) into carcinoma cell line (SK-CO-1) restored a normal β-catenin phenotype. This cell line has active Wnt signaling due to a homozygous APC $^{-/-}$ mutation. Moreover, such cells also did not demonstrate tumor formation when transferred *in vivo*. Vincan *et al., Differentiation* 2005; **73**: 142-153. This demonstrates that

the inhibition of Wnt signaling at the extracellular level can downregulate Wnt signaling resulting from activation of a downstream intracellular Wnt signaling pathway component. This further suggests that inhibitors such as the Wnt antagonists of the present invention, which inhibit Wnt-Frz interactions, have therapeutic benefit for any Wnt-mediated disorder, regardless of the particular manner in which Wnt signaling has been activated.

4. Aberrant Wnt signaling in colon cancer:

Defects in the Wnt signaling component APC was originally discovered to be the key in the hereditary cancer syndrome familial adenomatous polyposis (FAP). FAP patients who inherit one defective APC allele develop large number of colon polyps, or adenomas, in the early years of their life. Such polyps develop as clonal outgrowths of epithelial cells in which the second APC allele is inactivated. The cumulative effect of these FAP adenomas inevitably results in the appearance of adenocarcinomas, evident as a more or less ordered accumulation of mutations in additional oncogenes or tumor suppressor genes, such as K-Ras, p53 and Smad4. Moreover, the loss of APC also occurs in most sporadic colorectal cancers. Kinzler *et al., Cell* 87: 159-170 (1996). The mutational inactivation of APC, by resulting in the stabilization of, and eventual nuclear transport of β-catenin, and Wnt signaling, thereby transforms epithelial cells. Interestingly, reporter plasmids containing concatemerized TCF binding sites such as the pTOPFLASH, normally transcribed only upon Wnt signaling, are inappropriately transcribed in APC mutant cancer cells through constitutive activation of β-catenin/TCF-4 transcription complexes. In other examples of colorectal cancer in which *APC* in not mutated, the scaffolding protein Axin-2 is mutated [Liu *et al., Nature Genet.* 26: 146-147 (2000) or β-catenin is mutated so as to remove the N-terminal Ser/Thr destruction motif. [Morin *et al., Science* 275: 1787-1790 (1997). Thus, colorectal cancer is linked not only to defects in *APC*, but to the inappropriate persistence of β-catenin/TCF-4 transcriptional activation. It has further been reported that *TCF*-4 mutations result in activation of the same target genes (as shown by microarray analysis) in colorectal cancers, as is observed through defective APC expression in crypt stem and progenitor cells. Van de Wetering *et al., Cell* 111: 241-250 (2002). Once the Wnt cascade is activated, the $APC^{-/-}$ adenoma cells maintain their progenitor status indefinitely. As a result, it is likely that the activation of Wnt signaling is a necessary precursor in the carcinogenesis of colorectal cancer, and the inhibition of Wnt signaling could be an effective means to treat and/or prevent the onset of this disorder.

5. Wnt signaling in hematopoietic stem cells

Hematopoietic stem cells give rise to the adult blood cells of the circulatory system in a process of lineage-committed progenitor cells from multipotential hematopoietic stem cells (HSC). It is also apparent that Wnt signaling contributes to the self-renewal and maintenance of HSC's, and that dysfunctional Wnt signaling is responsible for various disorders resulting from HSC's, such as leukemias and various other blood related cancers. Reya *et al., Nature* 434: 843-850 (2005); Baba *et al., Immunity* 23: 599-609 (2005); Jamieson *et al., N. Engl. J. Med.* 351(7): 657-667 (2004). *Wnt* signaling is normally reduced as stem cells convert to committed myeloid progenitor cells. Reya *et al., Nature* 423: 409-414 (2003).

Not only are Wnt ligands themselves produced by HSC's, but Wnt signaling is also active, thereby suggesting autocrine or paracrine regulation. Rattis *et al., Curr. Opin. Hematol.* 11: 88-94 (2004); Reya *et al., Nature* 423: 409-414 (2003). Additionally, both β-catenin and Wnt3a promote self renewal of murine HSCs and progenitor cells, while application of Wnt-5A to human hematopoietic progenitors promotes the expansion of undifferentiated progenitors *in vitro*. Reya *et al., supra*.; Willert *et al., Nature* 423: 448-452 (2003); Van Den Berg *et al., Blood* 92: 3189-3202 (1998).

In addition to HSC's, it is apparent that embryonic stem cells, epidermal stem cells and epithelial stem cells are responsive or dependent on *Wnt* signaling for maintenance in an undifferentiated, proliferating state. Willert *et al., supra*; Korinek *et al., Nat. Genet.* 19: 379-383 (1998); Sato *et al., Nat. Med.* 10: 55-63 (2004); Gat *et al., Cell* 95: 605-614 (1998); Zhu *et al., Development* 126: 2285-2298 (1999). Therefore the inhibition of Wnt signaling with the Wnt antagonists of the present invention may be a therapeutic in the treatment of disorders resulting from dysfunctional hematopoieses, such as leukemias and various blood related cancers, such as acute, chronic, lymphoid and myelogenous leukemias, myelodysplastic syndrome and myeloproliferative disorders. These include myeloma, lymphoma (e.g., Hodgkin's and non-Hodgkin's) chronic and nonprogressive anemia, progressive and symptomatic blood cell deficiencies, polycythemia vera, essential or primary thrombocythemia, idiopathic myelofibrosis, chronic myelomonocytic leukemia (CMML), mantle cell lymphoma, cutaneous T-cell lymphoma, Waldenstrom macroglobinemia,

6. Wnt signaling in leukemia

Unregulated activation of the Wnt signaling pathway is a precursor to the development of leukemia. Reya *et al., supra*. Experimental evidence exists supporting the oncogenic growth of both myeloid and lymphoid lineages as dependent on Wnt signaling. Wnt signaling has been implicated in regulating both the chronic and acute forms of myeloid leukemia. Granulocyte-macrophage progenitors (GMPs) from chronic myelogenous leukemia patients and blast crisis cells from patients resistant to therapy display activated Wnt signaling. Jamieson, *et al., supra*. Moreover, inhibition of β-catenin through ectopic expression of Axin decreases the replating capacity of leukemic cells in vitro, suggesting that chronic myelogenous leukemia precursors are dependent on Wnt signaling for growth and renewal. Also, Wnt overexpression caused GMPs to acquire stem-cell-like properties of long-term self renewal. Jamieson *et al., supra.* This finding further support the hypothesis that Wnt signaling is necessary for the normal development of blood lineages, but that aberrant Wnt signaling results in the transformation of progenitor cells. The Wnt antagonists of the present invention would be useful to treat these types of leukemias.

Recent studies also suggest that lymphoid neoplasias may also be influenced by Wnt signaling. Wnt-16 is overexpressed in pre-B-cell leukemia cell lines carrying the E2A-PbX translocation, suggesting that autocrine Wnt activity may contribute to oncogenesis. McWhirter, *et al., Proc. Natl. Acad. Sci. USA* 96: 11464-11469 (1999). The role of Wnt signaling in the growth and survival of normal B-cell progenitors further supports this notion. Reya *et al., Immunity* 13: 15-24 (2000); Ranheim *et al., Blood* 105: 2487-2494 (2005). Autocrine dependence on *Wnt* has also been proposed for regulating the growth of multiple myeloma, a cancer of terminally differentiated B-cells. Derksen *et al., Proc. Natl. Acad. Sci. USA* 101: 6122-6127 (2004). Primary myelomas and myeloma cell lines were also found to express stabilized (i.e., independent of degradation complex). Although no mutations in Wnt signaling components was present, the overexpression of several components, including Wnt-5A and Wnt-10B suggest that tumor dependency and cancer self-renewal is not necessarily dependent on mutations appearing in Wnt signaling pathway components, but rather only upon constitutive activation of the pathway itself. Reya *et al., supra.* Through binding overexpressed *Wnt*, the *Wnt* antagonists of the present invention would be an effective therapeutic in treating B-cell leukemias.

The transition of self-renewing, pluripotent stem cells to myeloid progenitors is accompanied by the downregulation of Wnt signaling. Reya *et al, Nature* 423: 409-414

(2003). Similarly, the stable expression of β-catenin in lymphoid progenitors restored multiple differentiation options, albeit such cells lacked markers typically associated with either cell type. Baba *et al., Immunity* 23: 599-609 (2005). Thus, it is strongly suggested that the inhibition of Wnt signaling by the Wnt antagonists of the invention could be an effective therapeutic in treating leukemia, such as myelolid and lymphoid leukemia, including acute and chronic myelogenous leukemia as well as acute and chronic lymphoid leukemias.

7. Aberrant Wnt signaling in neural disorders

It has also been observed that the activation of Wnt signaling through β-catenin can increase cycling and expansion of neural progenitors, and that loss of such signaling can result in a loss of progenitor compartment. Chenn *et al., Science* 297: 365-369 (2002); Zechner *et al., Dev. Biol.* 258: 406-418 (2003). Just as normal activation of *Wnt* signaling may promote self-renewal of neuronal stem cells, aberrant Wnt pathway activation may be tumorigenic in the nervous system. Experimental evidence supporting this conclusion is the discovery that medulloblastoma, a pediatric brain tumor of the cerebellum, contains mutations in both β-catenin and Axin - thereby suggesting that medulloblastomas arise from primitive progenitors that become transformed in response to uncontrolled Wnt signaling. Zurawel *et al., Cancer Res.* 58: 896-899 (1998); Dahmen *et al., Cancer Res.* 61: 7039-7043 (2001); Baeza *et al., Oncogene* 22: 632-636 (2003). Thus, it is strongly suggested that the inhibition of Wnt signaling by the Wnt antagonists of the invention may be an effective therapeutic in the treatment of various neuronal proliferative disorders, including brain tumors, such as gliomas, astrocytomas, meningiomas, Schwannomas, pituitary tumors, primitive neuroectodermal tumors (PNET), medulloblastomas, craniopharyngioma, pineal region tumors, and non cancerous neurofibromatoses.

8. Aberrant Wnt signaling in breast cancer.

In mammary tissues where stem cells have yet to be definitively isolated, a controlling role for Wnt in progenitor cell fate or maintenance is suggested by studies of Wnt transgenic mice develop mammary tumors. These tumors have an increased frequency of individual cells with stem and progenitor properties, in stark contrast to tumors from mice overexpressing other oncogenes. [Liu *et al., Proc. Natl. Acad. Sci. USA* 101: 4158-4163 (2004); Li *et al., Proc. Natl. Acad. Sci. USA* 100: 15853-15858 (2003)]. This suggests that the Wnt pathway may be unique in its ability to target stem and progenitor cells for transformation, and suggests a key role in the self-renewal of normal breast epithelium.

Thus the inhibition of Wnt signaling by the Wnt antagonists of the invention is likely an effective therapeutic in the treatment of breast cancer.

Figure 32 is an illustration of active Wnt signaling in human breast cancer. Figure 32A shows Wnt-1 expression (as shown by *in vitro* hybridization) in normal (A-1), low grade (A-2) and high grade (A-3) human breast tumor initially reported in Wong *et al., J. Pathol.* 196: 145 (2002). Figure 32B shows nuclear (B-1) and cytoplasmic (B-2) localization (as shown by IHC) of β-catenin in breast cancer patients. Also shown is a Kaplan-Meier survival plot (B-3) showing patient survival probability that correlates with the indicated β-catenin expression pattern. This data was initially reported in Lin *et al., P.N.A.S. (USA)* 97(8): 4262-66 (2000). Figure 32C is a microarray analysis of Wnt-1 expression in a normal breast from a patient without cancer in comparison with tissue isolated from a patient with infiltrating ductal carcinoma, her-2 negative.

9. Wnt signaling in aging

The Wnt signaling pathway may also play a critical role in aging and age-related disorders.

As reported in Brack AS, *et al.*, *Science,* 317(5839):807-10 (2007), muscle stem cells from aged mice were observed to convert from a myogenic to a fibrogenic lineage as they begin to proliferate. This conversion is associated with an increase in canonical Wnt signaling pathway activity in aged myogenic progenitors and can be suppressed by Wnt inhibitors. Additionally, components of serum from aged mice bind to the Frizzled proteins and may account for the elevated Wnt signaling in aged cells. Injection of Wnt3A into young regenerating muscle reduced proliferation and increased deposition of connective tissue.

The Wnt signaling pathway has been further implicated in aging process in studies using the Klotho mouse model of accelerated aging in which it was determined that the Klotho protein physically interacted with and inhibited Wnt proteins. Liu H, et al., Science, 317(5839):803-6(2007). In a cell culture model, the Wnt-Klotho interaction resulted in the suppression of Wnt biological activity while tissues and organs from Klotho-deficient animals showed evidence of increased Wnt signaling.

Accordingly, Wnt antagonists could find use as therapeutics to reduce the effects of aging and to treat age-related diseases.

I. Modes of Administration Specific Formulations

1. General considerations

A pharmaceutical composition is formulated to be compatible with its intended route of administration, including intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (*i.e.,* topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

2. Injectable formulations

Pharmaceutical compositions suitable for injection include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, CREMOPHOR EL (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid so as to be administered using a syringe. Such compositions should be stable during manufacture and storage and must be preserved against contamination from microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (such as glycerol, propylene glycol, and liquid polyethylene glycol), and suitable mixtures. Proper fluidity can be maintained, for example, by using a coating such as lecithin, by maintaining the required particle size in the case of dispersion and by using surfactants. Various antibacterial and antifungal agents; for example, parabens, chlorobutanol, phenol, ascorbic acid, and thimerosal, can contain microorganism contamination. Isotonic agents; for example, sugars, polyalcohols such as manitol, sorbitol, and sodium chloride can be included in the composition. Compositions that can delay absorption include agents such as aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, any modulator substance/molecule of the invention) in the required amount in an

appropriate solvent with one or a combination of ingredients as required, followed by sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium, and the other required ingredients. Sterile powders for the preparation of sterile injectable solutions, methods of preparation include vacuum drying and freeze-drying that yield a powder containing the active ingredient and any desired ingredient from a sterile solutions.

3. Systemic administration

Systemic administration can also be transmucosal or transdermal. For transmucosal or transdermal administration, penetrants that can permeate the target barrier(s) are selected. Transmucosal penetrants include, detergents, bile salts, and fusidic acid derivatives. Nasal sprays or suppositories can be used for transmucosal administration. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams.

The compounds can also be prepared in the form of suppositories (*e.g.*, with bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

4. Carriers

In one embodiment, the active compounds are prepared with carriers that protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable or biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Such materials can be obtained commercially from ALZA Corporation (Mountain View, CA) and NOVA Pharmaceuticals, Inc. (Lake Elsinore, CA), or prepared by one of skill in the art. Liposomal suspensions can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, such as in (Eppstein et al., US Patent No. 4,522,811, 1985).

5. Unit dosage

Oral formulations or parenteral compositions in unit dosage form can be created to facilitate administration and dosage uniformity. Unit dosage form refers to physically discrete units suited as single dosages for the subject to be treated, containing a therapeutically effective quantity of active compound in association with the required pharmaceutical carrier. The specification for the unit dosage forms are dictated by, and

directly dependent on, the unique characteristics of the active compound and the particular desired therapeutic effect, and the inherent limitations of compounding the active compound.

6. Gene therapy compositions

The nucleic acid molecules can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (Nabel and Nabel, US Patent No. 5,328,470, 1994), or by stereotactic injection (Chen et al., *Proc Natl Acad Sci U S A.* 91:3054-7 (1994)). The pharmaceutical preparation of a gene therapy vector can include an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells that produce the gene delivery system.

7. Dosage

The pharmaceutical composition and method may further comprise other therapeutically active compounds that are usually applied in the administration of the Wnt antagonists.

In the treatment or prevention of conditions which require administration of Wnt antagonists, an appropriate dosage level will generally be about 0.01 to 500 mg per kg patient body weight per day which can be administered in single or multiple doses. Preferably, the dosage level will be about 0.1 to about 250 mg/kg per day; more preferably about 0.5 to about 100 mg/kg per day. A suitable dosage level may be about 0.01 to 250 mg/kg per day, about 0.05 to 100 mg/kg per day, or about 0.1 to 50 mg/kg per day. Within this range the dosage may be 0.05 to 0.5, 0.5 to 5 or 5 to 50 mg/kg per day. For oral administration, the compositions are preferably provided in the form of tablets containing 1.0 to 1000 milligrams of the active ingredient, particularly 1.0, 5.0, 10.0, 15.0, 20.0, 25.0, 50.0, 75.0, 100.0, 150.0, 200.0, 250.0, 300.0, 400.0, 500.0, 600.0, 750.0, 800.0, 900.0, and 1000.0 milligrams of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. The compounds may be administered on a regimen of 1 to 4 times per day, preferably once or twice per day.

However, the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that compound, the age,

body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the host undergoing therapy.

8. Kits for compositions

The compositions (*e.g.*, pharmaceutical compositions) can be included in a kit, container, pack, or dispenser together with instructions for administration. When supplied as a kit, the different components of the composition may be packaged in separate containers and admixed immediately before use. Such packaging of the components separately may permit long-term storage without losing the active components' functions.

Kits may also include reagents in separate containers that facilitate the execution of a specific test, such as diagnostic tests or tissue typing.

(a) *Containers or vessels*

The reagents included in kits can be supplied in containers of any sort such that the life of the different components are preserved and are not adsorbed or altered by the materials of the container. For example, sealed glass ampules may contain lyophilized modulator substance/molecule and/or buffer that have been packaged under a neutral, non-reacting gas, such as nitrogen. Ampules may consist of any suitable material, such as glass, organic polymers, such as polycarbonate, polystyrene, *etc.*, ceramic, metal or any other material typically employed to hold reagents. Other examples of suitable containers include simple bottles that may be fabricated from similar substances as ampules, and envelopes, that may consist of foil-lined interiors, such as aluminum or an alloy. Other containers include test tubes, vials, flasks, bottles, syringes, or the like. Containers may have a sterile access port, such as a bottle having a stopper that can be pierced by a hypodermic injection needle. Other containers may have two compartments that are separated by a readily removable membrane that upon removal permits the components to mix. Removable membranes may be glass, plastic, rubber, *etc.*

(b) *Instructional materials*

Kits may also be supplied with instructional materials. Instructions may be printed on paper or other substrate, and/or may be supplied as an electronic-readable medium, such as a floppy disc, CD-ROM, DVD-ROM, Zip disc, videotape, laserdisc, audio tape, *etc.* Detailed instructions may not be physically associated with the kit; instead, a user may be directed to an internet web site specified by the manufacturer or distributor of the kit, or supplied as electronic mail.

9. Combination therapy

In certain embodiments, a pharmaceutical formulation comprising a Wnt antagonist is administered in combination with at least one additional therapeutic agent and/or adjuvant. In certain embodiments, the additional therapeutic agent is a chemotherapeutic agent, growth inhibitory agent, or cytotoxic agent like a toxin, such as a maytansinoid, calicheamicin, antibiotic, radioactive isotope, nucleolytic enzyme or the like.

Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate formulations), and separate administration, in which case, administration of a Wnt antagonist can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant. A Wnt antagonist can also be used in combination with radiation therapy.

10. Medicaments

The invention provides a Wnt antagonist for a use in the preparation of a medicament useful for treating a Wnt-mediated disorder. In a specific aspect, the Wnt-mediated disorder is cancer.

The following examples are included to demonstrate preferred embodiments of the present invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention. All references cited throughout the specification are expressly incorporated by reference in their entirety herein.

Example 1

General Protocols

Mammalian Cell Culture.

Human kidney epithelial (HEK) 293 cells (ATCC # CRL-1573), human ovarian PA1 cells (ATCC # CRL-1572) were grown in 50/50 Dulbecco modified Eagle high glucose medium, Ham's F12 which has been supplemented with 10% fetal bovine serum. Human

teratoma derived NTer2 (ATCC #CRL-1973) and Tera2 (ATCC#HTB-106) cells were maintained in McCoy's medium supplemented with 15% fetal bovine serum and NCCIT cells (ATCC # CRL-2073) were maintained in RPMI supplemented with 10% fetal bovine serum. All cell lines were further supplemented with 2 mM glutamine, and 1% penicillin-streptomycin at 37°C in 5% CO_2.

Transfection and luciferase assays

In preparation for transfection, (1) 500,000 HEK293 and (2) 100,000 PA1 cells (ATCC # CRL-1572), NCCIT, NTera2 or Tera2 cells were plated into each well of a 12-well dish (Nuc) 24 hours before transfections. Cells were transfected with 0.375 μg TOPglow (Upstate, Cat # 21-204), 0.05 mg LEF1, 0.01 mg SV40 RL with Fugene (Roche) and at 24 hours post transfection. Media was changed and cells were untreated or treated with *Wnt3a* alone, *Wnt-5a* alone, or with serum samples for an additional 20-24 hours before harvesting. All dilutions were made in complete media for the indicated cell lines. Cells were harvested in 50-100 μl of 1XSJC lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 1mM EGTA, 1% Triton X-100, 10% Glycerol, 1.5 mM $MgCl_2$, 1 mM DTT, 50 mM NaF, 1 mM $NaVO_4$ and protease inhibitors) and duplicate 10 μl were assayed using Dual-Glo™ luciferase assay kit (Promega, Part # TM058) and detected in an Envision Luminometer (Perkin Elmer). Luciferase activity was normalized against *Renilla* activity.

Example 2

Construction of Frz-Fc chimeric molecules

Cloning and expression

Frz8(173)-Fc and Frz8(156)-Fc

Figures 4A and B show the sequences of the Frz8 (156)-Fc and Frz8 (173)-Fc chimeric constructs. Figure 4A shows the longer Frz8(173) sequence. Shown in gray (*i.e.*, first 24 N-terminal amino acid residues) is the leader signal sequence. Shown in underline (*i.e.*, residues 25-27) are alanine residues that may be present or absent in the mature protein. Shown in boxed text (*i.e.*, residues 157-173) are the additional sequences of the Frz8 receptors that distinguish the longer Frz8 (173) from the shorter Frz8(156) chimeric constructs. Shown in bold (*i.e.*, residues 174-182) is the linker sequence, while the sequence in italics (*i.e.*, residues 183-409) is the Fc region. Figure 4B shows the shorter Frz (156) minimal CRD (ECD) domain sequence. In gray (*i.e.*, first 24 N-terminal amino acid

residues) is the leader signal sequence. Shown in underline (*i.e.*, residues 25-27) are alanine residues that may be present or absent in the mature protein. Shown in bold (*i.e.*, residues 157-164) is the linker sequence, while the sequence in italics (*i.e.*, residues 165-391) is the Fc region.

The Frz8(156)-Fc construct was constructed as follows. cDNA encoding Frizzled 8 residues 1-156 were sub-cloned into the EcoR1 and XhoI sites of a pRK-derived plasmid. While native human cDNA is preferred, alternative sequence encoding identical protein sequence (e.g., murine) may also be used. In this cloning procedure, the carboxyl terminus of the Frz8 was fused to the amino terminus of a human IgG effector domain (Fc) via a short linker region (*e.g.*, residues LESGGGGVT)(SEQ ID NO: 70), to create an Frz8-Fc fusion. A final construct encodes 156 residues of Frz8. The cloning was performed using standard molecular biology techniques (Ausubel *et al.* (eds.), 2003, *Current Protocols in Molecular Biology*, 4 Vols., John Wiley & Sons). Protein was expressed in Chinese Hamster Ovary (CHO) cells.

Alternatively, the cDNA encoding a length of Frz8 of a length different than described previously (*e.g.*, 1-173) may be used. In addition, an alternative linker sequence (*e.g.*, ESGGGGVT)(SEQ ID NO: 69) may also be used.

Frz-Fc and sFrp-Fc constructs

The constructs for the Wnt antagonists with a Frizzled domain component comprising Frz1, Frz2, Frz3, Frz4, Frz5, Frz6, Frz7, Frz9, Frz10, sFRP1, sFRP2, sFRP3, sFRP4, or sFRP5 were constructed in a manner similar to the procedure described for Frz8. Frz2, Frz3, Frz4, Frz5, and sFRP3 were subcloned into a pRK-derived plasmid using XhoI and AscI. Frz1, Frz6, Frz7, Frz9, Frz10, and sFRP4 were subcloned into a pRK-derived plasmid using ClaI and XhoI and sFrp1, sFrp2, and sFRP5 were subcloned into a pRK-derived plasmid using ClaI and AscI. As with the Frz8 constructs, the carboxyl terminus of the Frz domains were fused to the amino terminus of a human IgG effector domain (Fc) via a short linker region to create the chimeric Wnt antagonists. Figure 7 (A, B, and C) shows exemplary amino acid sequences for these constructs. The leader signal sequence is shown in bold with italics indicating a non-native leader sequence. The linker is underlined and the Fc component is shown in italics.

Figure 5 (A-H) (SEQ ID NOs: 115-129) provides exemplary nucleic acid sequences for these Wnt antagonist constructs.

Alternative constructs can be made to optimize *in vivo* activity or stability or to provide other beneficial characteristics, such as, for example, increased solubility, improved binding characteristics. These constructs may include linkers that are different than the linkers of the above-described Wnt antagonists. For example, an alternative construct of the Frz3-Fc chimeric protein (SEQ ID NO: 114) has been made by subcloning a Frz3 domain into a pRK-derived plasmid using BstXI and XhoI and using the LESGGGGVT (SEQ ID NO: 70) peptide linker to fuse the Frz3 domain to the Fc domain.

Protein Isolation

The Wnt antagonist chimeric proteins were isolated to > 90% purity by affinity capture using a PROSEP® (Millipore) protein-A conjugated resin. Higher order aggregates were separated from dimers by passage over a Superdex 200® (GE-Healthcare) gel-filtration column. Protein identity and processing of the amino terminus to remove the signal sequence were confirmed by Edmund degradation. Purity of the final protein is estimated to be greater than 98% (Figure 10). Endotoxin levels of the material after purification is complete and less than 1.0 EU/mg.

Example 3

Serum Stability of Frz8-Fc chimeric molecules

Initial studies of the serum stability of the Frz8(173)-Fc chimeric constructs indicated that the construct had a limited *in vivo* half-life. The *in vivo* instability was likely due to the presence of protease cleavage sites in the EC domain (ECD) of the *Frizzled* receptor component. The Frz8(156)-Fc construct described in Example 2 exhibited increased serum stability over the Frz8(173)-Fc. Athymic nude mice were injected i.v. with 10 mg/kg of either Frz8(173)-Fc or Frz8(156)-Fc. Serum was collected at specified time points and analyzed for total and active protein. Figure 11A shows an immunoblot for human Fc used to detect the protein present in 1 μL of serum and compared with 25 μg of the respective purified protein (*P*). Frz8(156)-Fc was detectable in serum 72 h after administration, whereas Frz8(173)-Fc was not detectable beyond 30 minutes.

The activity of Frz8(156)-Fc and Frz8(173)-Fc in the collected serum was assayed by measuring the inhibition of Wnt3a-dependent TOPglow reporter activity in HEK293 cells. Although comparable *in vitro* potency was observed on treatment with purified Frz8(156)-Fc and Frz8(173)-Fc at 2.5 μg/mL, only partial inhibitory activity was recovered from the

serum of Frz8(173)-Fc-treated mice collected 30 minutes after protein administration. In contrast, more potent inhibitory activity could be recovered from the serum of Frz8(156)-Fc treated mice for up to 24 hours after administration, with detectable levels of inhibition for at least 72 hours (Figure 11B). These studies demonstrate that the Frz8(156) molecule is more stable *in vivo* than the molecule based on Frz8(173).

Additionally, the Frz8(173)-FC had suboptimal efficacy and acted only to reduce the rate of increase in tumor volume, as opposed to shrinking starting tumor volume. This suboptimal efficacy is illustrated in Figure 12, showing a graph of tumor volume over time resulting from treatment with various Wnt signaling component-Fc chimeric antagonists, including the Frz8(173)-FC molecule. In this assay, the MMTV-WNT-1 tumors were transplanted into the mammary fat pad of athymic nude mice, and drug was administered IV at the time points indicated by the arrows on the X-axis.

Example 4

***In vivo* pharmacokinetics of Frz8(156)-FC**

The *in vivo* pharmacokinetics of Frz8(156)-FC were tested by administration of a single dose of this protein at 1, 5, or 20 mg/kg i.v. or at 20 mg/kg i.p. into nude mice. As reported in Figure 13 and discussed further in this Example below, the Frz8-Fc reagent displayed biphasic elimination in nude mice at all doses. After a single IV or IP dose, Frz8-Fc displays: (1) dose proportional increase in exposure; (2) rapid absorption after IP dosing; (3) clearance of about 25-30 ml/day; and (4) a half life of about 4 days. Bioavailability coefficient, $AUC_{IP}/AUC_{IV} = 92\%$.

Animal protocol

Female athymic nude mice are separated into 4 groups of 12, on the basis of quantity of drug administered and manner of administration. Group 1: Frz8-Fc 1 mg/kg, intravenous (IV); Group 2: Frz8-Fc, 5 mg/kg, IV; Group 3: Frz8-Fc 20 mg/kg, IV; and Group 4: Frz8-Fc, 20 mg/kg, interperitoneal (IP). Each animal received an IV or IP bolus dose of Frz8-Fc according to the group designation. The dose volume administered (5-10 mL/kg) varies depending upon the concentration of the dosing solution and the weight of each animal. IV dosing is administered via the tail vein.

About 125 μl of blood is collected from each animal according to the following procedure. Serum is stored at -70°C until assayed by ELISA. Sample are drawn such that

n=3 animals/timepoint. Extra animals are used for predose sample collection and/or collection of blank mouse serum. Blood is collected with a retroorbital bleed for the first two timepoints for each animal, using alternative eyes. For the final timepoint, blood is collected via a cardiac stick and about 1 ml is aliquoted into 2 tubes. One sample will be used to determine Frz8-Fc concentration and the other will be reserved for research use. Each animal receives an IP bolus of 10 ml saline as fluid replacement after each blood collection timepoint. Retroorbital bleeds are performed under isoflorane anesthesia and terminal bleeds occur under a ketamine/xylazine cocktail. Animals are euthanized via cervical dislocation under anesthesia after the final blood draw.

Results

Figure 13A is an immunoblot of a neat serum from mice treated with Frz8-Fc showing detection in serum at 7 days and beyond from both 20 or 5 mg/kg I.V. or 20 mg/kg I.P. Samples were taken from individual mice at 4, 7, 10 or 14 days. For controls, serum samples were taken from untreated mice, Frz8-Fc protein was added to 20 μg/ml and the samples incubated for 2 hours at 37°C and the sample was then treated with SDS loading buffer (labeled as 2h); neat serum from untreated mice was also run as a negative control (labeled as S).

Figures 13B and 13C are a graphical summary of Frz8-Fc serum levels as determined from the pharmacokinetic study. Specific periods of time include evaluation over 16 days (Figure 13B) and 2 days (Figure 13C). Frz8-Fc displayed biphasic elimination administration in nude mice at all doses. Curves represent the predicted concentrations, while individual data points represent the average serum levels of Frz8-Fc protein from individual mice as determined by ELISA. Figure 13D is a summary of the parameters for a biphasic model of Frz8-Fc pharmacokinetics. When dosed at 20 mg/kg by either the i.p. or i.v. route, comparable serum levels of protein were achieved within a day of injection and the protein was detectable in serum up to 7 days. After i.p. dosing at 20 mg/kg, protein was rapidly absorbed with a T_{max} of ~8 h and bioavailability (AUC_{IP}/AUC_{IV}) of 92%. The clearance of the protein was ~25 to 30 mL/d/kg with a half-life of about 4 days

Example 5

Binding affinity of Frz-FC molecules

The addition of the FC domain to the Frz8(156) domains results in an increase in binding affinity for Wnt3a of over two magnitudes. Figure 14 demonstrates the enhanced ability of Frz8-ECD to block Wnt3a signaling when linked to a dimeric Fc domain. Figure 14A is an IC_{50} graph of a Wnt3a inhibition assay of two different preparations of Frz8(156)-FC. Figure 14B is a gel confirming the purity of the isolated Frz8(156) CRD (ECD). Shown are: (a) non-reduced Frz8(156) ECD (Lane 1); (b) molecular weight markers (Lane 2); and reduced Frz8 ECD (156) (Lane 3). This gel indicates that the Frz8 ECD used in the binding assay is intact and runs at approximately the expected molecular weight.

Example 6

Binding activity of Frz-FC chimeras

ELISA

For PK evaluation of the Wnt antagonist, the wells of a 384-well ELISA micro titer plate (Nunc Maxisorp, Rochester, NY) were coated with the rabbit anti-human Fc (Jackson Immuno Research, Westgrove, PA) at a concentration of 1 μg/ml in PBS (25 μg/well). After an overnight incubation at 4°C, the rabbit anti-human Fc solution was decanted, and the plates were blocked with 40 μl/well of block buffer (PBS containing 0.5% BSA and 10 ppm proclin). After a 60 minute incubation at room temperature with gentle agitation, the rabbit anti-human Fc coated plates were washed three times with wash buffer (PBS 0.05% Tween 20® and 10 ppm proclin). The Frizzled-Fc standards (a dilution series with a concentration range of 0.78 - 100 ng/ml), and the samples diluted into assay range in assay buffer (PBS containing 0.5% BSA, 0.05% Tween 20® and 10 ppm proclin) were added to the assay plate (25 μl/well). After a 120 minute incubation at room temperature with gentle agitation, the assay plates were washed six times with wash buffer. The remaining bound Frz-Fc was detected using a horse radish peroxidase (HRP) conjugated goat anti-human IgG-Fc (Jackson Immuno Research) diluted into assay diluent (25 μl/well). After appropriate color development (10-25 minutes) the enzymatic reaction was stopped with 1M phosphoric acid (25 μl/well). The assay plates were read at a wavelength of 450 nm with a reference wavelength of 630 nm. Sample concentrations were determined by comparing the sample OD against the standard curve fit using a 4-parameter algorithm.

BIAcore

Figure 15 demonstrates direct binding by Wnt3a to the Frz8(1–156)-Fc chimera. This chimera protein was amine coupled to a Biocore™ (BIAcore, Inc. Piscataway, N.J.) CM5 sensor chip at approximately 1700 response units as described generally in Chen, Y. *et al., J. Mol. Biol.* 293: 865-881 (1999). Briefly, carboxymethylated dextran biosensor chips (CM5, BIAcore™ Inc.) were activated with *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's instructions. An injection of 1M ethanolamine was done to block unreacted groups. *Wnt3a* was then injected at an estimated concentration of 0.5 µg/ml and binding was assessed by the change in response units as a function of time. Wnt3a was found to bind Frz8-Fc. As shown in Figure 15, the association of Wnt3a and Frz-Fc results in a highly significant increase of 1000 response units over control protein (E. coli expressed non-native Wnt3a.)

OCTET

The ability of the Wnt antagonists to interact with the Wnt ligands Wnt3a and Wnt5a was measured using the OCTET ™–QK system. (FortéBio, Inc., Menlo Park, CA). This system allows for the measurement of protein binding at a biosensor surface. The assays were conducted by first incubating one of the Wnt antagonist molecules (20 ug/mL) with anti-human IgG Fc-specific biosensors for 10 minutes in phosphate buffered saline (PBS) with 0.5% CHAPS. The unbound Wnt antagonist was removed by washing for 1.5 minutes in PBS 0.5% CHAPS. Either Wnt3a or Wnt5a (5.0ug/mL) was then added to the assay and incubated with the Wnt antagonist molecules bound to the biosensor surface for 5 minutes in PBS with 0.5% CHAPS. The interaction between the Wnt antagonist molecules and Wnt ligand was monitored in the same buffer. All assay steps were performed at room temperature in a volume of 150 uL. Figure 16 shows the result of this binding assay with Figure 16A showing data from the binding of Wnt3a to the Frz1-Frz10-Fc chimeras, Figure 16B showing data from the binding of Wnt3a to sFRP-Fc chimeras, and Figure 16C showing data from the binding of Wnt5a to the Frz1-Frz10-Fc chimeras and sFRP-Fc chimeras.

The OCTET ™ assay indicates that both Wnt3a and Wnt-5a bind Fz8-Fc, Fz5-Fc, and Fz4-Fc the fastest, relative to the other Frz proteins, with Wnt3a binding Fz1-Fc, Fz2-Fc, and Fz7-Fc at a slower rate. The amplitude and linear nature of Wnt-5a binding curves suggest a lower binding affinity relative to Wnt3a binding, as determined by this binding assay. The amplitude of the OCTET ™ binding data suggest that the sFRP-Fc proteins have

an affinity for Wnt3a similar that observed for Frz1, Frz2, and Frz7, and somewhat lower that observed for Frz5 and Frz8.

Example 7

Inhibition of Wnt Signaling by the Wnt Antagonists – cellular assays

Cellular assays were performed using 293 (human kidney) cells transfected with the TOPglow reporter plasmid. In preparation for transfection approximately 500,000 HEK293 were plated into a well of a 12-well dish (Nuc) 24 hours before transfections. Cells were transfected with 0.375 μg TOPglow (Upstate, Cat # 21-204), 0.05 mg LEF1, 0.01 mg SV40 RL with Fugene (Roche) and at 24 hours post transfection. Media was changed and cells were untreated or treated with Wnt3a alone, Wnt-5a alone, or with a Wnt antagonist for an additional 20-24 hours before harvesting. All dilutions were made in complete media for the indicated cell lines. Cells were harvested in 50-100 μl of 1XSJC lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 1mM EGTA, 1% Triton X-100, 10% Glycerol, 1.5 mM $MgCl_2$, 1 mM DTT, 50 mM NaF, 1 mM $NaVO_4$ and protease inhibitors) and duplicate 10 μl were assayed using Dual-Glo™ luciferase assay kit (Promega, Part # TM058) and detected in an Envision Luminometer (Perkin Elmer). Luciferase activity was normalized against *Renilla* activity.

Cells to be treated with Wnt5a were transfected with Frz4 and Lrp5 in addition to the reporter. The presence of these additional components allows Wnt pathway activation by Wnt5a to proceed as per the canonical pathway. Mikels AJ, and Nusse R., *PLOS Biol.* **4**:e115 (2006). Wnt3a activated cells were treated with 100ng/ml Wnt3a and Wnt5a activated cells were treated with 1ug/ml Wnt5a.

As shown in Figure 17A and B, the Frz-Fc antagonist inhibited Wnt signaling to varying degrees. Both Frz5-Fc and Frz8-Fc showed complete inhibition of the Wnt3a signal and significantly inhibited the Wnt5a signal. Frz4-Fc, Frz2-Fc, and Frz7-Fc showed significant inhibition of the Wnt3a signal.

Example 8

Relative IC50s of the Wnt Antagonists

The relative IC50s of the Wnt antagonists were determined by measuring inhibition of Wnt signaling by the Wnt antagonists in U2OS (human osteosarcoma) cells stably transfected with TOPglow luciferase TCF reporter plasmid as described in Example 7. Initial Wnt signaling in cells was obtained with Wnt3a activation. A 3-fold dilution series of Frz-Fcs was applied to cells overnight. (Figure 18). As determined by this assay, Wnt3a binds to Frz8-Fc, Frz5-Fc, and Frz4-Fc with sub-nanomolar IC50 (with Frz8-Fc having an IC50 of 0.04nM, Frz-5Fc having an IC50 of 0.20nM, and Frz-4 having an IC50 of 0.48 nM) and to Frz2-Fc and Frz7 –Fc with nanomolar IC50 (with Frz2-Fc having an IC50 of 1.2 nM and Frz2-Fc having an IC50 of 1.4 nM.

Example 9

Wnt target genes as pharmacodynamic markers of drug response.

As an alternative to immunohistochemical analysis of ß-catenin, transcriptional targets of Wnt were used to monitor inhibition of Wnt signaling activity. The cell lines that had autocrine Wnt signaling showed increased expression of known Wnt target genes and this expression was regulated by *in vitro* treatment with Wnt3a as well as by Frz8-Fc. RNA analysis of NTera-2 cells indicated that Frz8-Fc treatment affects expression of the Wnt target genes tested. Thus, the expression of these genes can be followed as an indicator of treatment efficacy. As an extension of these observations, expression of these Wnt target genes can be used as a diagnostic tool to identify cancers that are driven by Wnt signaling and are likely candidates for anti-Wnt therapeutic agents.

In vitro

In vitro comparative gene expression analysis on PA-1 cells treated with purified Wnt3a, Fz8 CRD-Fc, or a control protein was performed to determine the suitability of Wnt target genes to indicate *in vivo* inhibition of Wnt signaling in teratoma cells. RNA isolated from PA-1 cells that were treated with Wnt3a, Frz8-Fc, or control Fc protein was subject to microarray analysis and the change in expression levels of the indicated genes in response to exogenously added Wnt3a, Frz8-Fc, and control Fc protein was determined. For microarray analysis, cells were treated with the indicated proteins in triplicate and total RNA was isolated using the RNAeasy kit (Qiagen). Array analysis was done on the Affymetrix

Human Genome U133 Gene Chip set (Rubinfeld B, *et al.*, *Nat Biotechnol* 2006; 24:205–9). The specific probes and primer sets are shown in Figure 20.

The expression levels of previously identified targets of Wnt signaling such as Axin2, APCDD1, and Gad1 were up-regulated by Wnt3a treatment or down-regulated by Frz8-Fc treatment (Figure 19A). Moreover, some genes such as Lefty2 (A), Lefty1 (B), sFRP1, and Fzd5 were down-regulated by Wnt3a and up-regulated by inhibition of Wnt signaling with Frz8-Fc (Figure 19A). Subsequent gene expression analysis by qRT-PCR showed that these transcripts were similarly regulated by Wnt3a andFz8 CRD-Fc in NTera-2, Tera-2, and NCCIT cells as well.

In vivo

APCDD1, Gad1, and Fzd5 were among the most consistently modulated genes in above described *in vitro* analyses and were therefore selected as potential markers of Wnt responsiveness for *in vivo* tumor xenograft studies.

Tumor tissue RNA was purified from xenograft specimens collected at the end of the efficacy study and quantitative reverse transcription-PCR (qRT-PCR) analysis of Wnt-responsive transcripts carried out as previously described Rubinfeld B, *et al.*, *Nat Biotechnol* 2006; 24:205–9). Fold induction for each gene was determined using the ΔΔCt method and the result presented relative to glyceraldehyde-3-phosphate dehydrogenase. The specific probes and primer sets are shown in Figure 20. All reactions were done in duplicate and the average of at least two assays ± SEM was plotted.

Similar to the effects seen *in vitro*, treatment with therapeutic doses of Frz8-Fc reduced the expression of genes for APCDD1 and Gad1 and increased the expression of Fzd5 in tumors from the NTera-2 xenografts (Figure 19B). Although there is a general nonspecific down-regulation of all genes following CD4hFc treatment, these changes were not statistically significant compared with those seen in Frz8-Fc -treated tumors. These observations show that the antitumorigenic effects of Frz8-Fc *in vivo* are on target genes and that the expression levels of these genes can be used to monitor the efficacy of potential anti-Wnt therapeutic agents.

Example 10

Inhibitory effect of Wnt antagonists on growth of tumors in mice with allografts and human xenografts

The studies set forth in this Example indicate that the Wnt antagonists are useful in treating Wnt expressing tumors. The largely complete tumor regression in the case of Wnt-1-MMTV model illustrate the effect of the Wnt antagonists on tumors that are strongly Wnt driven. However, the significant effect of the Wnt antagonist on the PA-1 and NTer2 tumors also reflects the strong therapeutic potential to treat tumors that may not be entirely Wnt driven.

Animals

Female C57Bl6 mice (The Jackson Laboratory) were used for the passaging of MMTV-Wnt1 tumors. Maintenance of mice and in vivo procedures were carried out using Institutional Animal Care and Use Committee–approved protocols.

MMTV-Wnt model -allografts

Figure 21 is a linear schematic describing the vector construct used in the transfection to create the Wnt animal model. This construct mimics the constitutive Wnt signaling activation observed with MMTA viral insertion, as described in Tsujomoto *et al., Cell* 55: 619-625 (1988) and Li *et al., Oncogene* 19: 1002-1009 (2000).

Passaging of MMTV-Wnt1 transgenic tumors in mice.

The tumors from MMTV-Wnt1 transgenic mice were serially passaged in C57Bl6 mice for 6 to 10 passages by surgical implantation in the mammary fat pad. Tumor tissue was aseptically collected from the transgenic mouse, rinsed in HBSS and cut into small pieces. The recipient mice were anaesthetized with a mixture of ketamine (75–80 mg/kg) and xylazine (7.5–15 mg/kg), the tumor fragment inserted under the skin rostral to the third mammary fat pad, and the skin closed using wound clips. Tumors were passaged for a maximum of 10 passages, and after the first two passages, tumor tissue was examined histologically to confirm that it was of mammary origin and continues to express Wnt. Mammary adenocarcinomas develop in 6-12 months in the mice. Tumors isolated from these mice were used to create the transplant models described below.

In vivo studies.

For *in vivo* studies testing the efficacy of Wnt antagonists in the MMTV-Wnt model, the tumor cells were introduced by subcutaneous injection of cells obtained from macerated tumors tissue. Tumor tissue was aseptically collected from mice transplanted with tumors from *Wnt* transgenic mice (described above), rinsed in PBS or HBSS, cut into smaller pieces and macerated into HBSS using a cell dissociation kit (Sigma). The cells were washed twice in sterile HBSS and suspended in a 50% matrigel solution in HBSS. The cell suspension was inoculated subcutaneously into the mammary fat pad of athymic nude mice, with a volume not exceeding 150 μl/mouse.

For *in vivo* studies using the NTera2 or PA-1 animals models, cells were grown as described and harvested when growth is in the logarithmic phase. The cells were suspended in a 50% matrigel solution in HBSS and inoculated subsutaneously into athymic nude mice at a concentration of either 8 million cells/mouse (NTera2) or 10 million cells/mouse (PA-1).

Tumors were monitored daily and measured after 7-12 days of inoculation. Animals were separated into groups with identical mean tumor volumes in the range of 150-250 mm^3. Treatment with the Wnt antagonist started 1-2 days after grouping and the mice were dosed intraperitoneally (IP) or intravenously (IV) with 100-200 μl of Wnt antagonist, negative control protein CD4-Fc, or PBS negative control once daily. Subsequent drug treatments were repeated 2-3 times weekly and continued for 3-4 weeks. Tumor volume was measured twice weekly the animals were sacrificed when the tumor volume reached 2500 mm^3. Blood was collected during the study by an orbital vein bleed and the serum assayed for levels of therapeutic agent by SDS-PAGE followed by immunoblot and detection using HRP or fluorescent conjugated anti-human Fc, and for activity of the therapeutic agent by its ability to inhibit Wnt3a activation of TOPglow activity as described in Example 7.

Allograft Tumors

Inhibitory effect of Wnt antagonists on growth of tumor allografts

Treatment with Frz8-Fc by either the i.p. or i.v. routed resulted in rapid tumor regression with sustained inhibition during the course of treatment, whereas the negative control protein CD4-Fc had no effect relative to the PBS treatment. The treated mice were monitored for three weeks after termination of treatment and regrowth of tumors was eventually observed.

Figure 22 illustrates the efficacy of Frz8-Fc against MMTV-*Wnt* tumor transplants in athymic nude mice by intraperitoneal (IP) dosing. Figure 22A is a graph showing that nude mice hosting MMTV-Wnt-1 tumor transplants were administered PBS, CD4-Fc (10 mg/kg/day) or Frz8-Fc (10 mg/kg/day) by intraperitoneal injection twice weekly. Each group had 11 mice and the average tumor volume for the group was 226 mm^3 before the start of treatments. Mean tumor volume is plotted over time and the treatment days are indicated by arrows on the X-axis. On day 25, the control groups were sacrificed and the drug administration to the treatment group stopped. Figure 22B is tabular summary of mean tumor volume and mean % change in tumor volume over time in the four treatment groups. Note that in Figure 22B, the mean tumor volume after treatment with Frz8-Fc antagonist results in a reduction in tumor volume from 226mm to about 219 mm^3 on the fifth day after start of treatment, and about 67 mm^3 on the 18^{th} day. This represents a 4% and 70%, respectively, reduction in tumor size. In this study, tumors administered the Frz-Fc antagonist showed regression in tumor size compared with control animals. This demonstrates that Frz-Fc antagonists of the invention are tumoricidal as a single agent and are useful as anti-cancer therapeutics.

Figure 23 illustrates the efficacy of Frz8-Fc against MMTV-Wnt tumor transplant in athymic nude mice by intravenous (IV) dosing. Figure 23A is a graph showing that nude mice hosting MMTV-Wnt-1 tumor transplants were administered PBS, CD4-Fc (10 mg/kg/day) or Frz8-Fc (10 mg/kg/day) by intravenous injection three times weekly. Each group had 11 mice and the average tumor volume for the group was 226 mm^3 before the start of treatments. The fourth group (high bar) in this study included 10 mice with a mean tumor volume of 375 mm^3 at the start of the study that were treated with Frz8-Fc (10 mg/kg/day) by intravenous injection three times weekly. Mean tumor volume is plotted over time and the treatment days are indicated by arrows on the X-axis. On day 25, the control group animals were sacrificed and drug administration to the treatment group stopped. Figure 23B is a tabular summary of mean tumor volume and mean % change in tumor volume over time in the four treatment groups. Note that in all mice treated with Frz-Fc that the tumor burden was reduced from an average of 226 mm^3 to an average volume of 179 mm^3 on the 4^{th} day after start of treatment, and to 73 mm^3 after the 18^{th} day. This represents a 21% amd 67% reduction, respectively, in tumor volume. For the high bar group, tumor volume was reduced from an average of 376 mm^3 to 225 mm^3 on the 4^{th} day of treatment, and to 53 mm^3 on the 18^{th} day. This represents a 39% and 86% reduction, respectively, in tumor volume.

Inhibitory effect of serum obtained from treated mice on Wnt signaling

Inhibition of Wnt signaling from serum isolated from the treated mice is reported in Figure 24, with Figure 24A showing the results of serum isolated from IP treated mice, while the IV treated ones appear in Figure 24B. The data is presented as a bar graph showing the *Wnt* signaling antagonist activity in the TOPglow assay (as described in Example 7). The samples appear in groups according to treatment, mouse study number and dilution. The relative luciferase activity in the TOPglow gene reporter assay is shown on the Y-axis. All samples are treated with ~ 40 ng/ml purified *Wnt3a* except for NA (control). All other protein controls are present in the medium at 5 μg/ml.

Human xenograft tumors

Inhibition of naturally derived human tumor models by the Wnt antagonists would serve as a further indicator of their usefulness in treating human cancer. Human tumor–derived cell lines were tested for evidence of autocrine wnt signaling, similar to that seen in the PA-1 teratoma cell line, as an indication of usefulness in testing Wnt antagonist activity. The teratoma-derived NTera-2, Tera-2, and NCCIT cell lines exhibited basal Wnt signaling that could be inhibited by Frz8-Fc, in contrast with 293 cells that exhibited low basal signaling that was not inhibited by Frz8-Fc (Figure 25A). Nevertheless, all four teratoma cell lines seemed to express Wnt receptors, as signaling was further stimulated by Wnt3a treatment, which could be blocked by Frz8-Fc (Figure 25B). These results indicate that the teratoma cell lines express Wnt(s), which might contribute to their tumorigenicity. These lines were therefore evaluated for tumor formation in athymic nude mice and based on consistency of tumor formation, NTera-2 and PA-1 were selected for in vivo efficacy studies.

Inhibitory effect of Wnt antagonists on growth of NTera2 tumor xenografts

Treatment of mice exhibiting NTera2 tumor xenografts with the Wnt antagonist Frz8-Fc resulted in a reduction of tumor volume by approximately 50% and reduction tumor mass by approximately 70%, relative to the control mice.

Figure 26 shows the anti-tumor efficacy of Frz8-Fc treatment on the growth of NTera2 tumor xenografts in athymic nude mice. Athymic nude mice bearing NTera2 tumor xenografts were administered an initial dose of PBS, CD4-Fc and Frz8-Fc at 15 mg/kg/day, followed by subsequent doses of 10 mg/kg/day by intraperitoneal injection three times weekly. Each group had 20 mice and the average tumor volume for the group was 200 mm^3 before the start of treatments. The fourth group of the study included 10 mice with a mean tumor volume of 336 mm^3 at the start of the study that were treated with Frz8-Fc (10

mg/kg/day) by intraperitoneal injection three times weekly. Figure 26A is an exemplary procedural flow chart, while Figure 26B is a graph plotting mean tumor volume over time, wherein the treatment days are indicated by arrows on the X-axis. Figure 26C is a bar graph plotting the mean tumor weights at sacrifice of all animals in the group at day 20 of the study. Figures 26D and 26E are tabular summaries of mean tumor volume and mean % change in tumor volume, respectively.

Inhibitory effect of serum obtained from mice with NTera2 tumor xenografts on Wnt signaling

Figure 27 is a bar graph showing Wnt signaling antagonist activity in the TOPglow assay of the Frz8-Fc Wnt antagonist of serum isolated from various animals in the NTera2 tumor study. Relative luciferase activity (Y-axis) as measured from TOPglow assay from the controls and Frz8-Fc Wnt antagonist. No additional purified Wnt or Wnt conditioned media was added to the cells. These results demonstrate that reduced Wnt signaling is associated with reduction in tumor size in these mice treated with Frz8-Fc Wnt antagonist.

Inhibitory effect of Wnt antagonists on growth of PA-1 tumor xenografts

Treatment of mice exhibiting PA-1 tumor xenografts with the Wnt antagonist Frz8-Fc resulted in a significant reduction in tumor growth within 12 days of treatment. In this model, the tumors were approximately 50% smaller, with significantly smaller mass than tumors in the control mice at the end of the treatment period.

Figure 28 demonstrates the anti-tumor efficacy of Frz8-Fc treatment on the growth of PA-1 tumor xenografts in athymic nude mice. Athymic nude mice bearing PA-1 tumors xenografts were administered PBS, CD4-Fc or Frz-Fc at 15 mg/kg/day, followed by subsequent doses of 10 mg/kg/day by intraperitoneal injection three times weekly. Each group had 13 mice and the average tumor volume for the group was 168 mm^3 before the start of treatments. Figure 28A is an exemplary procedural flow chart, while Figure 28B is a graph plotting mean tumor volume over time, wherein the treatment days are indicated by arrows on the X-axis. Figure 28C is a graph of mean tumor weight at sacrifice. The mice were sacrificed on day 58 after cell inoculation (day 32 after start of treatments) and tumors were excised and weighed. The mean tumor weight ± SEM is plotted as a function of the group. Figures 28D and 28E are tabular summaries of mean tumor volume and mean % change in tumor volume, respectively.

Example 11

Wnt signaling in mice transplanted with MMTV tumors and treated with Frz8-Fc and Frz5-Fc *Wnt* antagonists

Effect of Frz8-Fc and Frz5-Fc Wnt antagonists on Wnt signaling

Frz5-Fz inhibits Wnt3a induced signaling as effectively as Frz8-Fc.

Athymic nude mice with MMTV tumors (approximately 400-800 cubic millimeters in size) were treated with Frz8-Fc, Frz5-Fz, or CD4-Fc, as a negative control, at 10mg/kg. Five hours after treatment, serum was collected by cardiac puncture from the mice and analyzed for Wnt inhibiting effect on 293 cells activated with Wnt3a and transfected with TOPglow as described in Example 7. All samples are treated with ~ 40 ng/ml purified Wnt3a except for NA (control). All other protein controls are present in the medium at 5 μg/ml. Figure 29 shows the level of inhibition in mice treated with Frz8-Fc or Frz5-Fz. Treatment with Frz8-Fc or Frz5-Fz resulted in similar levels of inhibition of Wnt 3a induced signaling.

Effect of Frz8-Fc and Frz5-Fc *Wnt* antagonists on Axin2 expression

Frz8-Fc and Frz5-Fz compounds inhibit *in vivo* Wnt signaling as determined by modulation of the Wnt target gene Axin2.

Athymic nude mice with MMTV tumors (approximately 400-800 cubic millimeters in size) were treated with Frz8-Fc, Frz5-Fz, or CD4-Fc, as a negative control, at 10mg/kg. Five hours after treatment, serum was collected by cardiac puncture from the mice. RNA was extracted from the tumor cells using the QIAGEN RNAEASY kit (Qiagen, Valencia, CA) and analyzed for expression of Axin2 as described in Example 9. Reduced levels of Axin2 was observed in samples obtained from mice treated with Frz8-Fc or Frz5-Fz indicating that these compounds are able to inhibit *in vivo* Wnt signaling. Figure 30 shows the reduced Axin2 expression in Frz8-Fc and Frz5-Fz treated tumor with Figure 30A showing expression normalized to expression of GAPDH and Figure 30B showing expression normalized to expression of rpl19.

Example 12

Regenerative Tissue treated with Wnt antagonist

Wnt signaling plays a critical role in self-renewal of regenerating tissue such as skin, intestine, and hematopoietic cells, and inhibition of Wnt signaling by Dkk1 can adversely affect the architecture of these tissues in adult mice. The following Example examines whether exposure to Frz8-Fc under the same conditions used to obtain antitumor efficacy had any effect on intestine and skin in the mice. Tissues were collected from mice that were treated in the MMTV-Wnt1 tumor model (described in Example 10) after 14 treatments, thrice a week, and sections were stained for ß-catenin protein by immunohistochemistry. Analysis of skin and various intestinal compartments revealed that the architecture of these tissues appeared morphologically normal in treated mice of all groups, with typical patterns of cytoplasmic and nuclear ß-catenin staining in intestinal Paneth cells (Figure 31A) and skin hair follicles (Figure 31B). Furthermore, histologic and immunohistochemical analysis of skin and intestine collected from animals using the NTera-2 model, after nine treatments, thrice a week also revealed no differences between control and treated groups. This suggests that treatment with Frz8-Fc with the therapeutic regimen that can inhibit tumor growth does not have adverse effects on tissue renewal of skin and intestine.

Example 13

This Example describes various methods of producing the Wnt antagonists.

Expression of Wnt Antagonist in *E. coli*

This example illustrates preparation of an unglycosylated form of Wnt antagonist by recombinant expression in *E. coli*.

The DNA sequence encoding Wnt antagonist is initially amplified using selected PCR primers. The primers should contain restriction enzyme sites which correspond to the restriction enzyme sites on the selected expression vector. A variety of expression vectors may be employed. An example of a suitable vector is pBR322 (derived from *E. coli*; see Bolivar *et al.*, Gene, 2:95 (1977)) which contains genes for ampicillin and tetracycline resistance. The vector is digested with restriction enzyme and dephosphorylated. The PCR amplified sequences are then ligated into the vector. The vector will preferably include sequences which encode for an antibiotic resistance gene, a trp promoter, a polyhis leader

(including the first six STII codons, polyhis sequence, and enterokinase cleavage site), the *Wnt* antagonist coding region, lambda transcriptional terminator, and an argU gene.

The ligation mixture is then used to transform a selected *E. coli* strain using the methods described in Sambrook *et al.*, supra. Transformants are identified by their ability to grow on LB plates and antibiotic resistant colonies are then selected. Plasmid DNA can be isolated and confirmed by restriction analysis and DNA sequencing.

Selected clones can be grown overnight in liquid culture medium such as LB broth supplemented with antibiotics. The overnight culture may subsequently be used to inoculate a larger scale culture. The cells are then grown to a desired optical density, during which the expression promoter is turned on.

After culturing the cells for several more hours, the cells can be harvested by centrifugation. The cell pellet obtained by the centrifugation can be solubilized using various agents known in the art, and the solubilized Wnt antagonist protein can then be purified using a metal chelating column under conditions that allow tight binding of the protein.

Wnt antagonist may be expressed in E. coli in a poly-His tagged form, using the following procedure. The DNA encoding Wnt antagonist is initially amplified using selected PCR primers. The primers will contain restriction enzyme sites which correspond to the restriction enzyme sites on the selected expression vector, and other useful sequences providing for efficient and reliable translation initiation, rapid purification on a metal chelation column, and proteolytic removal with enterokinase. The PCR-amplified, poly-His tagged sequences are then ligated into an expression vector, which is used to transform an *E. coli* host based on strain 52 (W3110 fuhA(tonA) lon galE rpoHts(htpRts) clpP(lacIq). Transformants are first grown in LB containing 50 mg/ml carbenicillin at 30°C with shaking until an O.D.600 of 3-5 is reached. Cultures are then diluted 50-100 fold into CRAP media (prepared by mixing 3.57 g $(NH_4)_2SO_4$, 0.71 g sodium citrate•2H2O, 1.07 g KCl, 5.36 g Difco yeast extract, 5.36 g Sheffield hycase SF in 500 mL water, as well as 110 mM MPOS, pH 7.3, 0.55% (w/v) glucose and 7 mM $MgSO_4$) and grown for approximately 20-30 hours at 30°C with shaking. Samples are removed to verify expression by SDS-PAGE analysis, and the bulk culture is centrifuged to pellet the cells. Cell pellets are frozen until purification and refolding.

E. coli paste from 0.5 to 1 L fermentations (6-10 g pellets) is resuspended in 10 volumes (w/v) in 7 M guanidine, 20 mM Tris, pH 8 buffer. Solid sodium sulfite and sodium

tetrathionate is added to make final concentrations of 0.1M and 0.02 M, respectively, and the solution is stirred overnight at 4°C. This step results in a denatured protein with all cysteine residues blocked by sulfitolization. The solution is centrifuged at 40,000 rpm in a Beckman Ultracentifuge for 30 min. The supernatant is diluted with 3-5 volumes of metal chelate column buffer (6 M guanidine, 20 mM Tris, pH 7.4) and filtered through 0.22 micron filters to clarify. The clarified extract is loaded onto a 5 ml Qiagen Ni-NTA metal chelate column equilibrated in the metal chelate column buffer. The column is washed with additional buffer containing 50 mM imidazole (Calbiochem, Utrol grade), pH 7.4. The protein is eluted with buffer containing 250 mM imidazole. Fractions containing the desired protein are pooled and stored at 4°C. Protein concentration is estimated by its absorbance at 280 nm using the calculated extinction coefficient based on its amino acid sequence.

The proteins are refolded by diluting the sample slowly into freshly prepared refolding buffer consisting of: 20 mM Tris, pH 8.6, 0.3 M NaCl, 2.5 M urea, 5 mM cysteine, 20 mM glycine and 1 mM EDTA. Refolding volumes are chosen so that the final protein concentration is between 50 to 100 micrograms/ml. The refolding solution is stirred gently at 4°C for 12-36 hours. The refolding reaction is quenched by the addition of TFA to a final concentration of 0.4% (pH of approximately 3). Before further purification of the protein, the solution is filtered through a 0.22 micron filter and acetonitrile is added to 2-10% final concentration. The refolded protein is chromatographed on a Poros R1/H reversed phase column using a mobile buffer of 0.1% TFA with elution with a gradient of acetonitrile from 10 to 80%. Aliquots of fractions with A280 absorbance are analyzed on SDS polyacrylamide gels and fractions containing homogeneous refolded protein are pooled. Generally, the properly refolded species of most proteins are eluted at the lowest concentrations of acetonitrile since those species are the most compact with their hydrophobic interiors shielded from interaction with the reversed phase resin. Aggregated species are usually eluted at higher acetonitrile concentrations. In addition to resolving misfolded forms of proteins from the desired form, the reversed phase step also removes endotoxin from the samples.

Fractions containing the desired folded *Wnt* antagonist polypeptide are pooled and the acetonitrile removed using a gentle stream of nitrogen directed at the solution. Proteins are formulated into 20 mM Hepes, pH 6.8 with 0.14 M sodium chloride and 4% mannitol by dialysis or by gel filtration using G25 Superfine (Pharmacia) resins equilibrated in the formulation buffer and sterile filtered.

Expression of Wnt Antagonist in mammalian cells

This example illustrates preparation of a potentially glycosylated form of *Wnt* antagonist by recombinant expression in mammalian cells.

The vector, pRK5 (see EP 307,247, published March 15, 1989), is employed as the expression vector. Optionally, the *Wnt* antagonist DNA is ligated into pRK5 with selected restriction enzymes to allow insertion of the *Wnt* antagonist DNA using ligation methods such as described in Sambrook *et al.*, supra. For purposes of this example, the resulting vector is referred to as pRK5-WA.

In one embodiment, the selected host cells may be 293 cells. Human 293 cells (ATCC CCL 1573) are grown to confluence in tissue culture plates in medium such as DMEM supplemented with fetal calf serum and optionally, nutrient components and/or antibiotics. About 10 μg pRK5-WA DNA is mixed with about 1 μg DNA encoding the VA RNA gene [Thimmappaya *et al.*, Cell, 31:543 (1982)] and dissolved in 500 μl of 1 mM Tris-HCl, 0.1 mM EDTA, 0.227 M $CaCl_2$. To this mixture is added, dropwise, 500 μl of 50 mM HEPES (pH 7.35), 280 mM NaCl, 1.5 mM $NaPO_4$, and a precipitate is allowed to form for 10 minutes at 25°C. The precipitate is suspended and added to the 293 cells and allowed to settle for about four hours at 37°C. The culture medium is aspirated off and 2 ml of 20% glycerol in PBS is added for 30 seconds. The 293 cells are then washed with serum free medium, fresh medium is added and the cells are incubated for about 5 days.

Approximately 24 hours after the transfections, the culture medium is removed and replaced with culture medium (alone) or culture medium containing 200 μCi/ml ^{35}S-cysteine and 200 μCi/ml ^{35}S-methionine. After a 12 hour incubation, the conditioned medium is collected, concentrated on a spin filter, and loaded onto a 15% SDS gel. The processed gel may be dried and exposed to film for a selected period of time to reveal the presence of *Wnt* antagonist polypeptide. The cultures containing transfected cells may undergo further incubation (in serum free medium) and the medium is tested in selected bioassays.

In an alternative technique, *Wnt* antagonist may be introduced into 293 cells transiently using the dextran sulfate method described by Somparyrac *et al.*, Proc. Natl. Acad. Sci., 12:7575 (1981). 293 cells are grown to maximal density in a spinner flask and 700 μg pRK5-WA DNA is added. The cells are first concentrated from the spinner flask by centrifugation and washed with PBS. The DNA-dextran precipitate is incubated on the cell pellet for four hours. The cells are treated with 20% glycerol for 90 seconds, washed with tissue culture medium, and re-introduced into the spinner flask containing tissue culture

medium, 5 μg/ml bovine insulin and 0.1 μg/ml bovine transferrin. After about four days, the conditioned media is centrifuged and filtered to remove cells and debris. The sample containing expressed *Wnt* antagonist can then be concentrated and purified by any selected method, such as dialysis and/or column chromatography.

In another embodiment, *Wnt* antagonist can be expressed in CHO cells. The pRK5-WA can be transfected into CHO cells using known reagents such as $CaPO_4$ or DEAE-dextran. As described above, the cell cultures can be incubated, and the medium replaced with culture medium (alone) or medium containing a radiolabel such as ^{35}S-methionine. After determining the presence of *Wnt* antagonist polypeptide, the culture medium may be replaced with serum free medium. Preferably, the cultures are incubated for about 6 days, and then the conditioned medium is harvested. The medium containing the expressed *Wnt* antagonist can then be concentrated and purified by any selected method.

Epitope-tagged *Wnt* antagonist may also be expressed in host CHO cells. The *Wnt* antagonist may be subcloned out of the pRK5 vector. The subclone insert can undergo PCR to fuse in frame with a selected epitope tag such as a poly-his tag into a Baculovirus expression vector. The poly-his tagged *Wnt* antagonist insert can then be subcloned into a SV40 driven vector containing a selection marker such as DHFR for selection of stable clones. Finally, the CHO cells can be transfected (as described above) with the SV40 driven vector. Labeling may be performed, as described above, to verify expression. The culture medium containing the expressed poly-His tagged *Wnt* antagonist can then be concentrated and purified by any selected method, such as by Ni^{2+}-chelate affinity chromatography.

Wnt antagonist may also be expressed in CHO and/or COS cells by a transient expression procedure or in CHO cells by another stable expression procedure.

Stable expression in CHO cells is performed using the following procedure. The proteins are expressed as an IgG construct (immunoadhesin), in which the coding sequences for the soluble forms (*e.g.* extracellular domains) of the respective proteins are fused to an IgG1 constant region sequence containing the hinge, CH2 and CH2 domains and/or is a poly-His tagged form.

Following PCR amplification, the respective DNAs are subcloned in a CHO expression vector using standard techniques as described in Ausubel *et al.*, Current Protocols of Molecular Biology, Unit 3.16, John Wiley and Sons (1997). CHO expression vectors are constructed to have compatible restriction sites 5' and 3' of the DNA of interest to allow the convenient shuttling of cDNA's. The vector used expression in CHO cells is as described in

Lucas *et al.*, Nucl. Acids Res. 24:9 (1774-1779 (1996), and uses the SV40 early promoter/enhancer to drive expression of the cDNA of interest and dihydrofolate reductase (DHFR). DHFR expression permits selection for stable maintenance of the plasmid following transfection.

Twelve micrograms of the desired plasmid DNA is introduced into approximately 10 million CHO cells using commercially available transfection reagents SUPERFECTt® (Quiagen), DOSPER® or FUGENE® (Boehringer Mannheim). The cells are grown as described in Lucas *et al.*, supra. Approximately 3 x 10^7 cells are frozen in an ampule for further growth and production as described below.

The ampules containing the plasmid DNA are thawed by placement into water bath and mixed by vortexing. The contents are pipetted into a centrifuge tube containing 10 mLs of media and centrifuged at 1000 rpm for 5 minutes. The supernatant is aspirated and the cells are resuspended in 10 mL of selective media (0.2 μm filtered PS20 with 5% 0.2 μm diafiltered fetal bovine serum). The cells are then aliquoted into a 100 mL spinner containing 90 mL of selective media. After 1-2 days, the cells are transferred into a 250 mL spinner filled with 150 mL selective growth medium and incubated at 37°C. After another 2-3 days, 250 mL, 500 mL and 2000 mL spinners are seeded with 3 x 10^5 cells/mL. The cell media is exchanged with fresh media by centrifugation and resuspension in production medium. Although any suitable CHO media may be employed, a production medium described in U.S. Patent No. 5,122,469, issued June 16, 1992 may actually be used. A 3L production spinner is seeded at 1.2 x 10^6 cells/mL. On day 0, the cell number pH is determined. On day 1, the spinner is sampled and sparging with filtered air is commenced. On day 2, the spinner is sampled, the temperature shifted to 33°C, and 30 mL of 500 g/L glucose and 0.6 mL of 10% antifoam (*e.g.*, 35% polydimethylsiloxane emulsion, Dow Corning 365 Medical Grade Emulsion) taken. Throughout the production, the pH is adjusted as necessary to keep it at around 7.2. After 10 days, or until the viability dropped below 70%, the cell culture is harvested by centrifugation and filtering through a 0.22 μm filter. The filtrate was either stored at 4°C or immediately loaded onto columns for purification.

For the poly-His tagged constructs, the proteins are purified using a Ni-NTA column (Qiagen). Before purification, imidazole is added to the conditioned media to a concentration of 5 mM. The conditioned media is pumped onto a 6 ml Ni-NTA column equilibrated in 20 mM Hepes, pH 7.4, buffer containing 0.3 M NaCl and 5 mM imidazole at

a flow rate of 4-5 ml/min. at 4°C. After loading, the column is washed with additional equilibration buffer and the protein eluted with equilibration buffer containing 0.25 M imidazole. The highly purified protein is subsequently desalted into a storage buffer containing 10 mM Hepes, 0.14 M NaCl and 4% mannitol, pH 6.8, with a 25 ml G25 Superfine (Pharmacia) column and stored at -80°C.

Immunoadhesin (Fc-containing) constructs are purified from the conditioned media as follows. The conditioned medium is pumped onto a 5 ml Protein A column (Pharmacia) which had been equilibrated in 20 mM Na phosphate buffer, pH 6.8. After loading, the column is washed extensively with equilibration buffer before elution with 100 mM citric acid, pH 3.5. The eluted protein is immediately neutralized by collecting 1 ml fractions into tubes containing 275 μL of 1 M Tris buffer, pH 9. The highly purified protein is subsequently desalted into storage buffer as described above for the poly-His tagged proteins. The homogeneity is assessed by SDS polyacrylamide gels and by N-terminal amino acid sequencing by Edman degradation.

Expression of Wnt Antagonist in Yeast

The following method describes recombinant expression of *Wnt* antagonist in yeast.

First, yeast expression vectors are constructed for intracellular production or secretion of *Wnt* antagonist from the ADH2/GAPDH promoter. DNA encoding *Wnt* antagonist and the promoter is inserted into suitable restriction enzyme sites in the selected plasmid to direct intracellular expression of *Wnt* antagonist. For secretion, DNA encoding *Wnt* antagonist can be cloned into the selected plasmid, together with DNA encoding the ADH2/GAPDH promoter, a native *Wnt* antagonist signal peptide or other mammalian signal peptide, or, for example, a yeast alpha-factor or invertase secretory signal/leader sequence, and linker sequences (if needed) for expression of *Wnt* antagonist.

Yeast cells, such as yeast strain AB110, can then be transformed with the expression plasmids described above and cultured in selected fermentation media. The transformed yeast supernatants can be analyzed by precipitation with 10% trichloroacetic acid and separation by SDS-PAGE, followed by staining of the gels with Coomassie Blue stain.

Recombinant *Wnt* antagonist can subsequently be isolated and purified by removing the yeast cells from the fermentation medium by centrifugation and then concentrating the medium using selected cartridge filters. The concentrate containing *Wnt* antagonist may further be purified using selected column chromatography resins.

Expression of Wnt Antagonist in Baculovirus-Infected Insect Cells

The following method describes recombinant expression of *Wnt* antagonist in Baculovirus-infected insect cells.

The sequence coding for *Wnt* antagonist is fused upstream of an epitope tag contained within a baculovirus expression vector. Such epitope tags include poly-his tags and immunoglobulin tags (like Fc regions of IgG). A variety of plasmids may be employed, including plasmids derived from commercially available plasmids such as pVL1393 (Novagen). Briefly, the sequence encoding *Wnt* antagonist or the desired portion of the coding sequence of *Wnt* antagonist such as the sequence encoding an extracellular domain of a transmembrane protein or the sequence encoding the mature protein if the protein is extracellular is amplified by PCR with primers complementary to the 5' and 3' regions. The 5' primer may incorporate flanking (selected) restriction enzyme sites. The product is then digested with those selected restriction enzymes and subcloned into the expression vector.

Recombinant baculovirus is generated by co-transfecting the above plasmid and BACULOGOLD™ virus DNA (Pharmingen) into *Spodoptera frugiperda* ("Sf9") cells (ATCC CRL 1711) using lipofectin (commercially available from GIBCO-BRL). After 4 - 5 days of incubation at 28°C, the released viruses are harvested and used for further amplifications. Viral infection and protein expression are performed as described by O'Reilley *et al.*, Baculovirus expression vectors: A Laboratory Manual, Oxford: Oxford University Press (1994).

Expressed poly-his tagged *Wnt* antagonist can then be purified, for example, by Ni^{2+}-chelate affinity chromatography as follows. Extracts are prepared from recombinant virus-infected Sf9 cells as described by Rupert *et al.*, Nature, 362:175-179 (1993). Briefly, Sf9 cells are washed, resuspended in sonication buffer (25 mL Hepes, pH 7.9; 12.5 mM $MgCl_2$; 0.1 mM EDTA; 10% glycerol; 0.1% NP-40; 0.4 M KCl), and sonicated twice for 20 seconds on ice. The sonicates are cleared by centrifugation, and the supernatant is diluted 50-fold in loading buffer (50 mM phosphate, 300 mM NaCl, 10% glycerol, pH 7.8) and filtered through a 0.45 μm filter. A Ni^{2+}-NTA agarose column (commercially available from Qiagen) is prepared with a bed volume of 5 mL, washed with 25 mL of water and equilibrated with 25 mL of loading buffer. The filtered cell extract is loaded onto the column at 0.5 mL per minute. The column is washed to baseline A_{280} with loading buffer, at which point fraction collection is started. Next, the column is washed with a secondary wash buffer (50 mM phosphate; 300 mM NaCl, 10% glycerol, pH 6.0), which elutes

nonspecifically bound protein. After reaching A_{280} baseline again, the column is developed with a 0 to 500 mM Imidazole gradient in the secondary wash buffer. One mL fractions are collected and analyzed by SDS-PAGE and silver staining or Western blot with Ni^{2+}-NTA-conjugated to alkaline phosphatase (Qiagen). Fractions containing the eluted His_{10}-tagged *Wnt* antagonist are pooled and dialyzed against loading buffer.

Alternatively, purification of the IgG tagged (or Fc tagged) Wnt antagonist can be performed using known chromatography techniques, including for instance, Protein A or protein G column chromatography.

Purification of Wnt Antagonist Polypeptides Using Affinity Chromatography

Native or recombinant Wnt Antagonist polypeptides may be purified by a variety of standard techniques in the art of protein purification. For example, pro-, mature, or pre-Wnt antagonist polypeptide is purified by immunoaffinity chromatography using antibodies specific for the Wnt antagonist polypeptide of interest. In general, an immunoaffinity column is constructed by covalently coupling the Wnt antagonist polypeptide to an activated chromatographic resin. Alternatively, Wnt antagonist which contain an Fc domain may be purified directly from media using a immobilized protein A resin such as ProSepA (Millipore).

Polyclonal immunoglobulins are prepared from immune sera either by precipitation with ammonium sulfate or by purification on immobilized Protein A (Pharmacia LKB Biotechnology, Piscataway, N.J.). Likewise, monoclonal antibodies are prepared from mouse ascites fluid by ammonium sulfate precipitation or chromatography on immobilized Protein A. Partially purified immunoglobulin is covalently attached to a chromatographic resin such as CnBr-activated SEPHAROSE™ (Pharmacia LKB Biotechnology). The antibody is coupled to the resin, the resin is blocked, and the derivative resin is washed according to the manufacturer's instructions.

Such an immunoaffinity column may be utilized in the purification of Wnt antagonist polypeptide by preparing a fraction from cells containing Wnt antagonist in a soluble form. This preparation is derived by solubilization of the whole cell or of a subcellular fraction obtained via differential centrifugation by the addition of detergent or by other methods well known in the art. Alternatively, soluble Wnt antagonist polypeptide containing a signal sequence may be secreted in useful quantity into the medium in which the cells are grown.

A soluble Wnt antagonist polypeptide-containing preparation is passed over the immunoaffinity column, and the column is washed under conditions that allow the preferential absorbance of Wnt antagonist polypeptide (e.g., high ionic strength buffers in the presence of detergent). Then, the column is eluted under conditions that disrupt antibody/Wnt antagonist binding (e.g., a low pH buffer such as approximately pH 2-3, or a high concentration of a chaotrope such as urea or thiocyanate ion), and Wnt antagonist polypeptide is collected.

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CLAIMS

1. A Wnt antagonist comprising:

(a) a Frizzled domain component, and

(b) a Fc domain,

wherein the Frizzled domain component comprises a polypeptide derived from a protein selected from the group consisting of (i) a Frizzled (Frz) protein, (ii) a secreted Frizzled Related Protein (sFRP) protein, and (iii) a Ror protein, and

further wherein the Wnt antagonist is active *in vivo* for at least 1 hour.

2. The Wnt antagonist of Claim 1, wherein the Wnt antagonist is active *in vivo* for at least 5 hours.

3. A Wnt antagonist comprising:

(a) a Frizzled domain component, and

(b) a Fc domain,

wherein the Frizzled domain component comprises a polypeptide derived from a protein selected from the group consisting of (i) a Frizzled (Frz) protein, (ii) a secreted Frizzled Related Protein (sFRP) protein, and (iii) a Ror protein, and

further wherein the Wnt antagonist has an *in vivo* half-life of at least 1 day.

4. The Wnt antagonist of Claim 3, wherein the Wnt antagonist has an *in vivo* half-life of at least 2 days.

5. The Wnt antagonist of any of Claims 1-4, wherein the Frizzled domain component comprises a minimal CRD (ECD) domain from a Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 18), hFrz2 (SEQ ID NO: 19), hFrz3 (SEQ ID NO: 20), hFrz4 (SEQ ID NO: 21), hFrz5 (SEQ ID NO: 22), hFrz6 (SEQ ID NO: 23), hFrz7 (SEQ ID NO: 24), hFrz8 (SEQ ID NO: 25), hFrz9 (SEQ ID NO: 26), and hFrz10 (SEQ ID NO: 27), and active variants thereof.

6. The Wnt antagonist of any of Claims 1-4, wherein the Frizzled domain component comprises a minimal CRD (ECD) domain from a sFRP polypeptide selected from the group

consisting of sFRP1 (SEQ ID NO: 28), sFRP2 (SEQ ID NO: 29), sFRP3 (SEQ ID NO: 30), sFRP4 (SEQ ID NO: 31), and sFRP5 (SEQ ID NO: 32), and active variants thereof.

7. The Wnt antagonist of any of Claims 1-4, wherein the Frizzled domain component comprises a minimal CRD (ECD) domain from a Ror polypeptide selected from the group consisting of hRor1 (SEQ ID NO: 33), and hRor2 (SEQ ID NO: 34), and active variants thereof.

8. The Wnt antagonist of any of Claims 1-4, wherein the Frizzled domain component comprises a mature Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 50), hFrz2 (SEQ ID NO: 51), hFrz3 (SEQ ID NO: 52), hFrz4 (SEQ ID NO: 53), hFrz5 (SEQ ID NO: 54), hFrz6 (SEQ ID NO: 55), hFrz7 (SEQ ID NO: 56), hFrz8 (SEQ ID NO: 57), hFrz9 (SEQ ID NO: 58), and hFrz10 (SEQ ID NO: 59), and active variants thereof.

9. The Wnt antagonist of any of Claims 1-4, wherein the Frizzled domain component comprises a mature sFrp polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 60), sFRP2 (SEQ ID NO: 61), sFRP3 (SEQ ID NO: 62), sFRP4 (SEQ ID NO: 63), and sFRP5 (SEQ ID NO: 64), and active variants thereof.

10. The Wnt antagonist of any of Claims 1-4, wherein the Frizzled domain component comprises a mature Ror polypeptide selected from the group consisting of hRor1 (SEQ ID NO: 65), and hRor2 (SEQ ID NO: 66), and active variants thereof.

11. The Wnt antagonist of any of Claims 1-4, wherein the Frizzled domain component comprises a pro-Frz polypeptide selected from the group consisting of hFrz1 (SEQ ID NO: 35), hFrz2 (SEQ ID NO: 36), hFrz3 (SEQ ID NO: 37), hFrz4 (SEQ ID NO: 38), hFrz5 (SEQ ID NO: 39), hFrz6 (SEQ ID NO: 40), hFrz7 (SEQ ID NO: 41), hFrz8 (SEQ ID NO: 42), hFrz9 (SEQ ID NO: 43), and hFrz10 (SEQ ID NO: 44), and active variants thereof.

12. The Wnt antagonist of any of Claims 1-4, wherein the Frizzled domain component comprises a pro-sFrp polypeptide selected from the group consisting of sFRP1 (SEQ ID NO: 45), sFRP2 (SEQ ID NO: 46), sFRP3 (SEQ ID NO: 47), sFRP4 (SEQ ID NO: 48), and sFRP5 (SEQ ID NO: 49), and active variants thereof.

13. The Wnt antagonist of any of Claims 1-12, wherein the Fc component is derived from an immunoglobulin selected from the group consisting of IgG1, IgG2, IgG3 and IgG4.

14. The Wnt antagonist of Claim 13, wherein the Fc is derived from an IgG1 immunoglobulin.

15. The Wnt antagonist of Claim 14, wherein the Fc comprises the Fc sequence shown in SEQ ID NO: 67 or SEQ ID NO: 68.

16. The Wnt antagonist of any of Claims 1-15, further comprising a linker connecting the Frizzled domain component to the Fc domain.

17. The Wnt antagonist of Claim 16, wherein the linker comprises a peptide selected from the group consisting of ESGGGGVT (SEQ ID NO: 69), LESGGGGVT (SEQ ID NO: 70), GRAQVT (SEQ ID NO: 71), WRAQVT (SEQ ID NO: 72), and ARGRAQVT (SEQ ID NO: 73).

18. A Wnt antagonist comprising a polypeptide selected from the group consisting of Frz8-Fc (SEQ ID NO: 74), Frz5-Fc (SEQ ID NO: 75), Frz1-Fc (SEQ ID NO: 76), Frz2-Fc (SEQ ID NO: 77), Frz3-Fc (SEQ ID NO: 78), Frz4-Fc (SEQ ID NO: 79), Frz6-Fc (SEQ ID NO: 80), Frz7-Fc (SEQ ID NO: 81), Frz9-Fc (SEQ ID NO: 82), Frz10-Fc (SEQ ID NO: 83), sFRP1-Fc (SEQ ID NO: 84), sFRP2 (SEQ ID NO: 85), sFRP3-Fc (SEQ ID NO: 86), sFRP4-Fc (SEQ ID NO: 87), and sFRP5-Fc (SEQ ID NO: 88).

19. A composition comprising at least one pharmaceutically acceptable carrier or excipient and any of the Wnt antagonists of Claims 1-18.

20. A nucleic acid sequence encoding any of the Wnt antagonists of Claims 1-18.

21. The nucleic acid of Claim 20 further comprising a vector containing control sequences to which the nucleic acid is operably linked.

22. The vector of Claim 21 further comprising a host cell.

23. The host cell of Claim 22 which is selected from the group consisting of a mammalian, insect, *E. coli* and yeast cell.

24. An article of manufacture comprising the composition of Claim 19 and a container, wherein the Wnt antagonist is contained within the container and the container further comprises (a) a label affixed to the container, or (b) a package insert inside the container referring to the use of the Wnt antagonist indicating use of the composition for the therapeutic treatment or diagnostic detection of a Wnt-mediated disorder.

25. A method of inhibiting Wnt signaling in a cell comprising contacting the cell with an effective amount of the any of the Wnt antagonists of Claims 1-18.

26. The method of Claim 25, wherein the cell is contained within a mammal and the amount administered is a therapeutically effective amount.

27. The method of Claim 25, wherein the Wnt signaling results from activation of a Wnt signaling component through somatic mutation.

28. The method of Claim 25, wherein the inhibition of Wnt signaling results in the inhibition of growth of the cell.

29. The method of Claim 28, wherein the cell is a cancer cell.

30. A method of treating a Wnt-mediated disorder in a mammal suffering therefrom, comprising administering to the mammal a therapeutically effective amount of a Wnt antagonist according to any of Claims 1-18.

31. The method of Claim 30, wherein the disorder is a cell proliferative disorder associated with aberrant Wnt signaling activity.

32. The method of Claim 31, wherein the aberrant Wnt signaling activity results from increased expression of a Wnt protein.

33. The method of Claim 31, wherein the cell proliferative disorder is cancer.

34. The method of Claim 33, wherein the cancer is selected from the group consisting of colon cancer, colorectal cancer, breast cancer, leukemia, gliomas, and medulloblastomas.

35. A method for detecting the presence of a Wnt protein, comprising contacting the sample with a Wnt antagonist according to any of Claims 1-18, wherein (a) the presence of a complex, or (b) the binding level between the Fz/Fc antagonist and Wnt protein is indicative of the presence of a Wnt protein and/or signaling.

36. The method of Claim 35, wherein the method further comprises determining if the level of Wnt signaling is aberrant, the method further comprising comparing the level of binding in the sample to the level in a second sample known to have physiologically normal *Wnt* signaling, wherein a level of binding in the sample that is higher or lower than that of the second sample is indicative of aberrant *Wnt* signaling.

37. The method of Claim 36, wherein the aberrant Wnt signaling is further indicative of the presence of a Wnt-mediated disorder.

38. The method of Claim 37, wherein the Wnt-mediated disorder is cancer.

39. A method of modulating the expression of a Wnt target gene in a cell characterized by activated or excessive Wnt signaling, comprising contact the cell with an effective amount of a Wnt antagonist of any of Claims 1-18.

40. A method of therapeutically treating a Wnt-mediated cancer, comprising administering a therapeutically effective amount of a Wnt antagonist of any of Claims 1-18, wherein the administration of the antagonist arrests any subsequent increase in size or advancement in severity of the cancer.

41. The method of Claim 40, wherein the administration of the Wnt antagonist results in the reduction in size or severity of the cancer.

42. The method of Claim 40, wherein the administration of the Wnt antagonist reduces the tumor burden of the cancer.

43. The method of Claim 40, wherein the administration of the Wnt antagonist kills the cancer.

44. Use of a Wnt antagonist in the manufacture of a medicament for the treatment of a cell proliferative disorder, the Wnt antagonist comprising:

(a) a Frizzled domain component, and

(b) a Fc domain,

wherein the Frizzled domain component comprises a polypeptide derived from a protein selected from the group consisting of (i) a Frizzled (Frz) protein, (ii) a secreted Frizzled Related Protein (sFRP) protein, and (iii) a Ror protein, and

further wherein the Wnt antagonist is active *in vivo* for at least 1 hour.

45. The use of Claim 44, wherein the Wnt antagonist is active *in vivo* for at least 5 hours.

46. Use of a Wnt antagonist in the manufacture of a medicament for the treatment of a cell proliferative disorder, the Wnt antagonist comprising:

(a) a Frizzled domain component, and

(b) a Fc domain,

wherein the Frizzled domain component comprises a polypeptide derived from a protein selected from the group consisting of (i) a Frizzled (Frz) protein, (ii) a secreted Frizzled Related Protein (sFRP) protein, and (iii) a Ror protein, and

further wherein the Wnt antagonist has an *in vivo* half-life of at least 1 day.

47. The use of Claim 46, wherein the Wnt antagonist has an *in vivo* half-life of at least 2 days.

48. The use of any of Claims 44-47 further comprising a linker connecting the Frizzled domain component to the Fc domain.

49. The use of Claim 48, wherein the linker is peptide selected from the group consisting of ESGGGGVT (SEQ ID NO: 69), LESGGGGVT (SEQ ID NO: 70), GRAQVT (SEQ ID NO: 71), WRAQVT (SEQ ID NO: 72), and ARGRAQVT (SEQ ID NO: 73).

50. The use of any of Claims 44-49, wherein the cell proliferative disorder is cancer.

51. The use of Claim 50, wherein the cancer is selected from the group consisting of colon cancer, colorectal cancer, breast cancer, leukemia, gliomas, and medulloblastomas.