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14. ABSTRACT Wnts are lipid-bound glycoproteins that can regulate diverse biological processes in many different cell systems. I have found that Wnt-5a, a member of the Wnt family unable to stabilize cytosolic β -catenin in endothelial cells and, in consequence, a noncanonical Wnt, is endogenously expressed in human primary endothelial cells as well as closely related member 5b. Overexpression of Wnt-5a in human endothelial cells was able to induce cell proliferation and enhance survival under serum-deprived conditions. Regulation of in vitro cell proliferation was blocked by addition of purified Frizzled-4 extracellular domain, showing evidence that this Frizzled, a receptor know to trigger canonical Wnt signaling response, can also mediate alternative Wnt signaling. Cells ectopically overexpressing Wnt-5a were also able to show improved network formation capabilities when cultured on top of extracellular matrix proteins. After a DNA array analysis screening a large number of target genes, we confirmed that in human microvascular cells, alternative Wnt signaling can upregulate expression of matrix metalloproteinase-1 (MMP-1), an interstitial collagenase, both at message and protein expression level.					
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Introduction

Several of the Wnt genes family are expressed and differentially regulated during breast development. The Wnt signal transduction pathway has been implicated in mammary tumorigenesis in different species as well as in human breast cancer cell lines. The various Wnt family members can exert differential effects on the growth and morphogenesis of mammary epithelial cells, while some Wnts are strongly transforming factors, others show weak or directly no transforming effect at all. Wnts are known to activate several different signaling pathways through the same receptor and thereby elicit unique cellular responses. These pathways include the activation of β -catenin/Tcf/Lef axis (canonical or classical Wnt signaling) and the noncanonical Wnt pathways which signal independently of β -catenin. Although a large amount of evidence links noncanonical Wnts with development of various forms of cancer, including breast cancer, its function and regulation is yet mostly unexplored territory. In consequence, new findings in this area can be a contribution of great interest to cancer research.

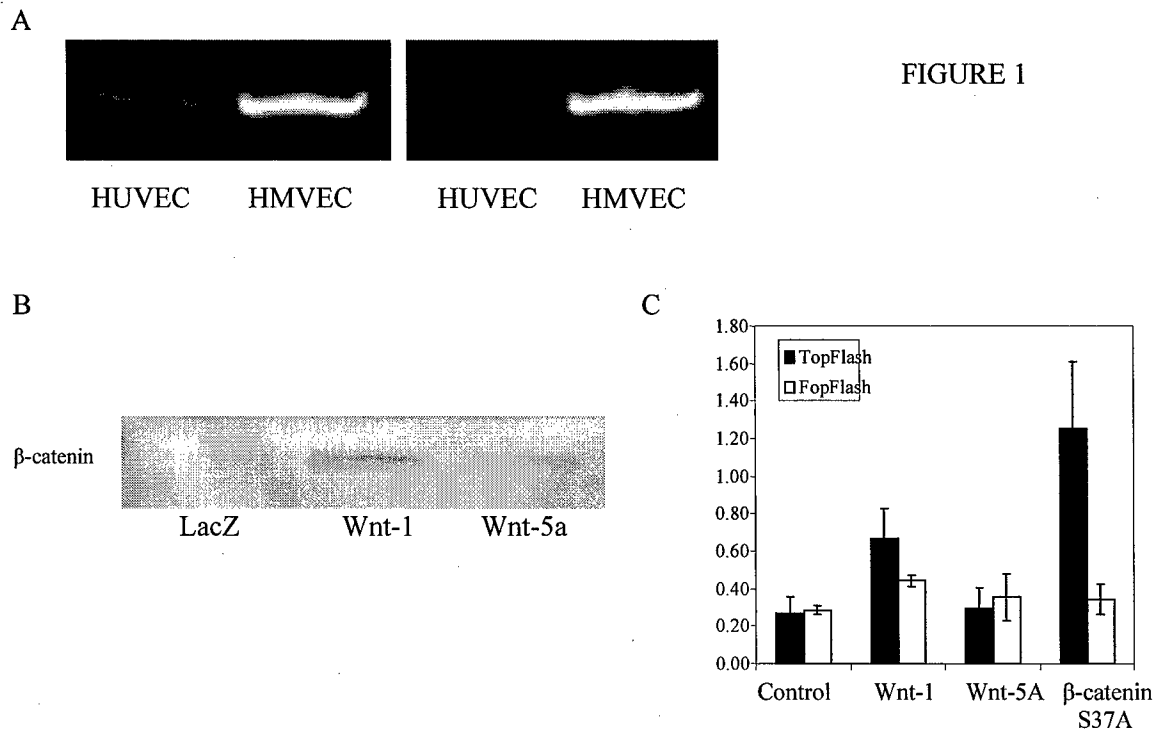
Important Note – Change in the SOW: In the last two years, new and exciting data has pointed the importance of Wnt signaling in angiogenesis. This includes the discovery of a new non-Wnt ligand of Frizzled receptors (Norrin, the protein product found mutated in Norrie Disease (ND) patients) and the finding that mutations in Wnt receptor Frizzled-4 or coreceptor LRP-5 are underlying molecular defects directly linked to Familial Exudative Vitreoretinopathy (FEVR) (Xu et al, 2004; Toomes et al, 2004). Both ND and FEVR are disorders with dysfunctional vasculature as a main characteristic. Here I report that noncanonical/alternative Wnt signaling plays a significant functional role in human vascular biology. In addition to this, results using breast cancer cells in culture were not as initially expected (no significant changes in JNK or Rho-GTPases activity were detected after overexpression of Wnt-5a in the breast cancer cell lines tested). In consequence, a new Statement of Work was submitted to the DOD with the approval of the project's Mentor and the Office of Grants and Contracts at Columbia University, although the original project title was left unchanged as instructed. Basically, the new Statement of Work is still entirely focused on alternative Wnt signaling but, for the reasons listed above, work was changed for research on human primary endothelial cells. We believe this new area has strong relevance for research in angiogenesis and, in consequence, in breast cancer. Thus, the proposed focus for the project would explore the importance of alternative Wnt signaling in angiogenesis using human primary endothelial cells. A copy of the modified Statement of Work was included in this report in **Appendix 1**.

Report Body

A series of *in vitro* functional assays to evaluate *in vitro* angiogenesis were developed. These assays employ the adenoviruses and constructs previously developed during year 1 of this fellowship. As primary endothelial cells can only be poorly transfected with plasmids and commercially available transfection reagents, the use of retroviral lines or adenovirus infections to overexpress proteins is crucial. As suggested by reviewers of the first annual report for this project, a description of the materials and methods used was added as **Appendix 2**.

Wnt-5a is endogenously expressed in human endothelial cells and is a noncanonical Wnt

As evaluated by RT-PCR, expression of either Wnt-5a or closely related Wnt-5b was detected in primary endothelial cells from both a large vessel (HUVEC) and microvasculature (HMVEC) (Figure 1A).



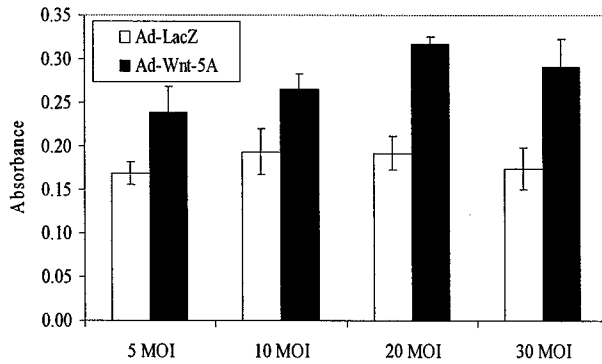
Infection of HUVEC with adenovirus encoding to overexpress Wnt-1 (a known canonical Wnt), Wnt-5a or control LacZ gene confirmed that Wnt-5a is unable to stabilize cytosolic β -catenin in human primary endothelial cells by Western blot (Figure 1B). In accordance with this, a transfection of a reporter construct (TopFlash-luciferase) that respond to canonical Wnt signaling activation or control FopFlash-luciferase (with mutated Tcf/Lef binding sites) both showed no response to Wnt-5a or control LacZ adenovirus, while it showed the expected response to either Wnt-1 or a mutant β -catenin with a longer half life in cytosols (β -cateninS37A).

Wnt-5a can induce endothelial cell proliferation

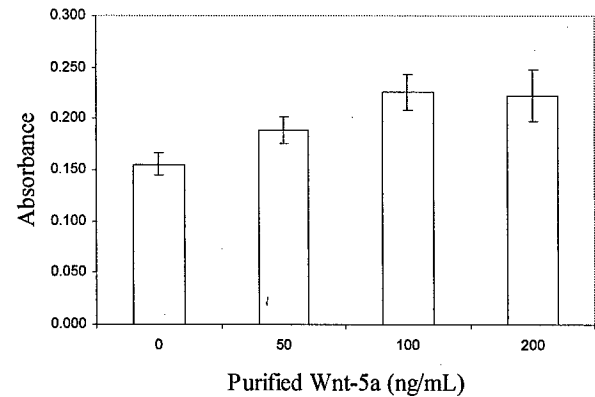
To analyze the biological effects of Wnt-5a overexpression in human endothelial cells, adenoviral infected HUVEC were assayed for proliferation. Adenoviral infected HUVEC overexpressing Wnt-5a were incubated for 48 to 72 hours in presence of serum-free endothelial medium added with bFGF showed increased proliferation when compared to LacZ control cells (Figure 2A). The response was dose-dependent up to 20 MOI. This response correlates well with what was observed when HUVEC were incubated under the same conditions without any infection but in presence of purified Wnt-5a protein in a dose-dependent manner up to 100 ng/mL (Figure 2B). In a parallel experiment, a portion of Fzd-4 extracellular domain (Fzd-4-CRD) was used as a competitive inhibitor of Wnt-5a in proliferation assays with adenoviral infected HUVEC to overexpress Wnt-5a or control gene LacZ (Figure 2C), a condition that blocked Wnt-5a-induced proliferation.

FIGURE 2

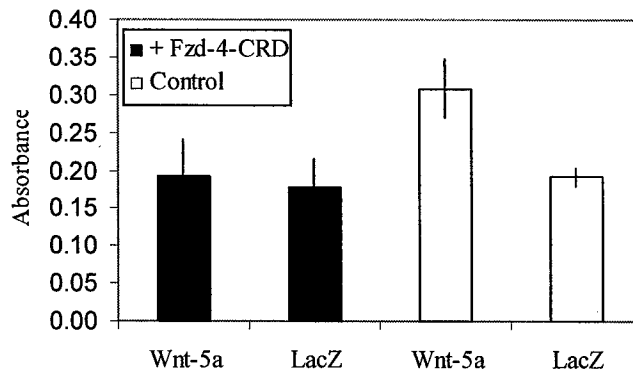
A



B



C



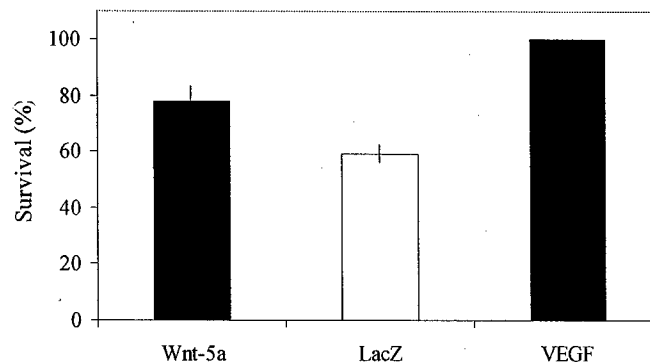
Wnt-5a shows ability to enhance HUVEC survival and network formation

Effects on endothelial cell survival were analyzed using adenoviral infected HUVEC and enhanced survival was observed in infected cells overexpressing Wnt-5a as compared to control (LacZ) but less than cells cultured in presence of control cells incubated with VEGF (Figure 3A).

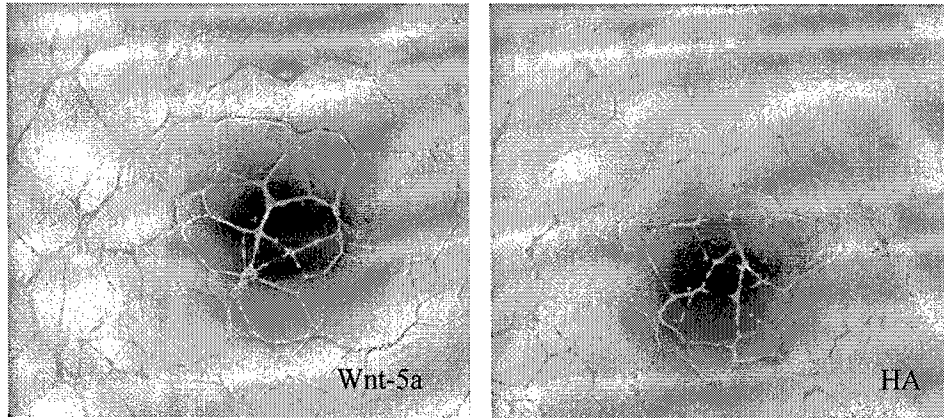
The ability of endothelial cells to form a network of capillaries after they migrate from a pre-existing blood vessel is a crucial property in both normal and tumor angiogenesis. We further analyzed the ability of HUVEC cultured on top of extracellular matrix components to form a network-like structure when they were either overexpressing Wnt-5a or their endogenous expression of this gene was decreased. After 18-19 hours of incubation, cells showed a better network when they were overexpressing Wnt-5a-HA by retroviral infection and selection as compared to control (HA) (Figure 3B).

FIGURE 3

A



B



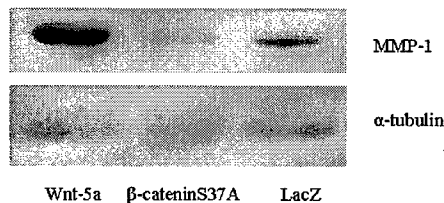
Alternative Wnt signaling can induce MMP-1 expression in endothelial cells in vitro

To better understand the potential mechanisms by which Wnt-5a signaling would promote endothelial cell proliferation, survival and improved network formation on top of extracellular matrix gels, a screening of genes differentially regulated by Wnt-5a versus control LacZ gene was performed in endothelial cells after adenoviral infection. The ectopic expression of Wnt-5a was confirmed by Western blot (not shown) and results were compared using a value of $p < 0.05$ to select genes of interest. Data from this DNA array analysis is still under analysis, and validation of target genes important in angiogenesis is currently being done. So far, matrix metalloproteinase-1 (MMP-1) is one of the genes that was selected to validate whether it can be regulated by noncanonical Wnt signaling in human primary endothelial cells. MMP-1 is an interstitial collagenase shown to be overexpressed in many tumors. I have found that MMP-1 expression was upregulated by Wnt-5a in endothelial cells by RT-PCR analysis, Western blot and an ELISA-based activity assay (Figures 4A, B and C, respectively). In these experiments, adenoviral overexpression of β -cateninS37A was unable to upregulate MMP-1, showing that this effect is exclusive for alternative/noncanonical Wnt signaling.

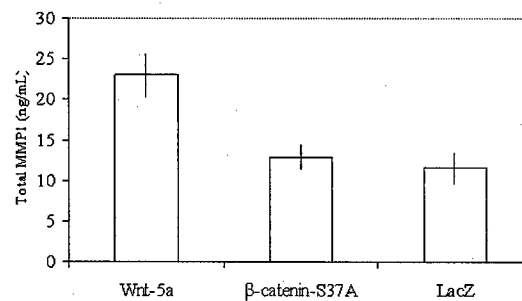
A



B



C



Key research accomplishments/Reportable outcomes

During this period, significant findings were achieved in showing that alternative Wnt signaling does play a role in angiogenesis. After the confirmation that Wnt-5a is a noncanonical/alternative Wnt in human primary endothelial cells, previously unknown biological and gene regulatory effects were found when this Wnt is overexpressed. The fact that Frizzled-4 showed evidence that is a receptor for Wnt-5a in human primary endothelial cells acquires special importance as this Frizzled has become directly involved in angiogenesis in at least two known human syndromes (FEVR and Norrie Disease). Our finding that Frizzled-4 extracellular domain can block Wnt-5a-induced proliferation of endothelial cells *in vitro* is consistent with experimental evidence showing that Frizzled-4 and Wnt-5a can specifically interact. In 293 cells, Frizzled-4 endocytosis depends on added Wnt-5a protein, a process also involving phosphorylated Dishevelled-2, a well-known downstream mediator of Wnt signaling (Chen et al, 2003). Phosphorylation of Dishevelled-2 and -3 after Wnt-5a stimulation is currently being investigated.

MMP-1, an interstitial collagenase, might play an important role in the biology of certain tumors, as immunocytochemical expression of MMP-1 was found to be the strongest of all MMPs in tumors including soft tissue neoplasms, fibromatoses and sarcomas of diverse grade (Roebuck et al, 2005). Endogenous expression at various levels of endogenous expression of MMP-1 can be detected in primary microvascular endothelial cells from humans (Plaisier et al, 2004). Wnt-5a has already been linked to regulation of a matrix metalloproteinase, as overexpression of Wnt-5a and rat Frizzled-2 in C57mg mammary epithelial cells is able to upregulate expression of stromelysin-1 (MMP-3) (Prieve et al, 2003).

The finding that overexpression of Wnt-5a can upregulate MMP-1 *in vitro* is a first evidence of the role of endogenous Wnt-5a in human endothelial cells and might be important in the understanding of the role of noncanonical Wnt signaling in tumor angiogenesis. Current work is being performed to validate other target genes and to successfully silence the Wnt-5a gene using shRNA (short hairpin RNA) in order to confirm biological findings.

A research article was submitted to *Angiogenesis* and, after being reviewed favorably, is currently in press (see Abstract in **Appendix 3**). Also, a poster was presented at the Era of Hope Meeting (Philadelphia, PA, June 8-11, 2005) (see **Appendix 4**). Currently, two more publications also with the PI as a first author are under preparation for submission to scientific journals, as well as a communication for a Wnt Signaling meeting in September. All these publications and presentations acknowledge the DOD and the present award.

Conclusions

- Wnt-5a is a non canonical Wnt in human primary endothelial cells, which endogenously express Wnt members 5a and 5b at different levels in microvascular or venous endothelial cells.
- Alternative Wnt signaling can induce proliferation, cell survival and network formation in human primary endothelial cells.
- Experimental evidence strongly supports the idea that Frizzled-4 is a receptor for Wnt-5a and plays a role in the induction of proliferation in human endothelial cells.
- Alternative Wnt signaling can upregulate expression of MMP-1 at the gene message and protein level.

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STATEMENT OF WORK

"Activation of alternative Wnt signaling pathways in human mammary gland and breast cancer cells".

Task 1. To evaluate in vitro changes in the activity of Rho-GTPases and JNK in human mammary gland and breast cancer cell lines.

- a. To develop and purify plasmids for expressing Wnts, Frizzled receptors, dishevelleds, axin, Rho-GTPases and JNK in mammalian cells (months 1- 4).
- b. Set up of assays for Rho-GTPases and JNK and of transfections into a variety of human immortalized epithelial mammary gland cells and breast cancer cell lines (months 5 - 9).
- c. Screening of activity for Rho-GTPases and JNK in the previously tested cells (months 10 - 19).
- d. Analysis of biological effects of alternative/non-canonical Wnt-signaling in human endothelial cells in culture by overexpression of Wnts (months 20 – 26).

Task 2. Analysis of gene expression in response to induction of non-canonical Wnt signaling.

- a. Development of DNA array analysis to screen for target genes regulated by non-canonical Wnt signaling (months 27 - 28).
- b. Validation (RT-PCR and Western blots/activity assays) of selected genes detected by the previous DNA array analysis (months 29 - 30).
- c. Development and use of shRNA probes to interfere with alternative/non-canonical Wnt signaling in human cells and analysis of biological effects in vitro (months 31 -33).
- d. Analysis of the interactions between non-canonical and canonical Wnt signaling (months 34 – 36).

Appendix 2

Materials and Methods

Cells and reagents

HUVEC were isolated from human umbilical vein as previously described (Jaffe et al, 1973) and grown in EGM2 (BioWhittaker) with VEGF, bFGF, EGF, IGF-1 and 2% FBS on dishes coated with Type I rat tail collagen (Upstate Biotechnology, USA). HMVEC and their media were obtained from BioWhittaker, Inc. (USA). Adenoviruses encoding for Wnts or mutant β -catenin were prepared as described (Young et al, 2003). Anti- β -catenin antibody was purchased from Upstate Biotechnology Inc. and SuperTopFlash and SuperFopFlash Tcf luciferase reporter constructs were generously provided by Dr. Randall Moon (University of Washington School of Medicine, Seattle, WA).

Gene transfer into HUVEC

To infect cells, 4×10^5 passage 3-5 cells were trypsinized and resuspended in 300 μ l full culture medium. Adenovirus stock was added at 30 MOI unless indicated otherwise for each experiment, and cells were incubated at 37°C for 1 hour with gentle shaking every 10 min. Then, cells were seeded onto type I collagen-coated plates and harvested 24-48 hours later depending on the assay. For retroviral gene transfer, the retroviral vector pHyTCX was used. 5.0×10^6 GP293 packaging cells (Clontech) were seeded, transfected with 10 μ g pHyTC-genes and pVSVG and retroviral-containing supernatants were collected 48 hr later. Retroviruses were added to passage 4 HUVEC and 48 hrs later cells were selected for 4-5 days with hygromycinB at 300 μ g/ml and then maintained with hygromycinB at 100 μ g/ml. An expression vector encoding the HA-tag (pHyTC-HA) was used as negative controls. Protein expression in HUVEC was evaluated by immunoblotting using antibodies to the HA epitope in order to proper expression of Wnt.

Reporter assays

Cells were seeded on collagen-coated 24-well plates (33,000 cells per well). The next day, cells were transfected with either Tcf/Lef transcriptional activation reporter construct SuperTopFlash containing Tcf responsive elements or SuperFopFlash with mutated elements (control) and a renilla-luciferase construct in all wells. Transfections were performed in triplicates using 0.18 μ g of reporter plasmid, 0.02 μ g of renilla-luciferase plasmid and 0.45 μ g of inducer plasmid in total with 1.3 μ l of Lipofectin in OptiMEM media (Invitrogen, USA) per well following manufacturer's instructions for procedure. Cells were incubated at 37°C for 5 hours with transfection cocktail and then were incubated overnight with fresh full endothelial culture media. Cell lysates were prepared the next day and both firefly and renilla luciferase activities were evaluated using Dual-Luciferase Reporter Assay System (Promega, USA). Samples were read in a luminometer and values were normalized for transfection efficiency using renilla-luciferase activity.

Proliferation and survival assays in vitro

Low-passage primary cells were split and suspended in full endothelial culture media. Cells were infected by incubation with Wnt-adenoviruses as described above, using adenovirus encoding for the LacZ gene as a negative control. Next, cells were seeded at 10,000 cells/well on 24-well plates with 0.5 mL of culture media that were pre-collagen-coated. Cells were allowed to seed overnight and media was then replaced with basal endothelial cell media EBM-2 added with bFGF. Blocking experiment with Frizzled-4-CRD was performed incubating cells under the same conditions but adding purified Frizzled-4-CRD (R&D Systems Inc, MN) at 2 ng/ μ l. Cell numbers at 48 hours post-incubation with basal media were assessed with the Cell Counting Kit-8 assay (Dojindo, USA). All assays were performed in quadruplicate.

Endothelial network formation assay

Retroviral selected HUVEC expressing Wnt-5a-HA or control gene (HA tag) were analyzed by Western blot to confirm the overexpression of the desired protein. Matrigels were prepared using 24-well plates with 0.3 mL pure Growth Factor-reduced Matrigel (BD Biosciences) per well and incubating plates at 37°C for 1 hour. Retroviral selected HUVEC grown on collagen-coated plates were seeded at 100,000 cells per well on top of Matrigel in the presence of 0.8 mL of full EC culture medium. Pictures were taken with 4x magnification after 18-19 hours of incubation at 37°C. All experiments were performed in duplicates and repeated twice to confirm results.

RT-PCR and DNA array analysis

Low passage human primary endothelial cells were cultured in EGM-2 BulletKit (BioWhittaker, Inc.). Total RNA was isolated using RNeasy Protect kit (Qiagen, Germany), reverse transcription reaction was performed using Omniscript Reverse Transcriptase as described by manufacturer's instructions (Qiagen, Germany) and PCR was done using Platinum Taq DNA Polymerase (Invitrogen, Inc.). Conditions for Wnt-5a and Wnt-5b PCR were 94°C 3 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min and 72 °C for 1 min plus 2 min at 72 °C. Primers used were 5'GTGCAATGTCTTCCAAGTTCTTC 3' forward and 5'GGCACAGTTTCTTCTGTCCTTG 3' reverse for Wnt-5a, and 5' GACGCCAACTCCT GGTGGC 3' forward and 5'GCATGACTCTCCCAAAGACAGATG 3' reverse for Wnt-5b while product sizes were 195 bp and 258 bp, respectively. PCR for MMP-1 was performed using R&D Systems Inc., MN primer pair following manufacturer's instructions. To correct for sample variations in RT-PCR efficiency, β -actin expression was used to normalize the RNA samples using the following primers: 5' CGAGGCCAGAGCAAGAGAG 3' upper primer; 5' CTCGTAGATGGGCACAG TGTG 3' lower primer with a product size of 336 bp. Conditions for β -actin PCR were 94°C 2 min followed by 20 cycles of 94°C for 45 sec, 60°C for 1 min and 72 °C for 1 min plus 5 min at 72 °C. DNA array analysis was performed with cDNA obtained from HUVEC adeno-infected to express either Wnt-5a or LacZ gene (control) at 48 hours post-infection. Hu95Av2 GeneChips, which query about 10,000 genes, were purchased from Affymetrix (Santa Clara, CA). Microarray and probes were prepared and used as described before (Li et al, 2002). To analyze data, for each sample, the signals for each gene (probe set) were normalized to the values of the entire microarray and statistical analyses were done using GeneSpring TM Software (Silicon Genetics).

Activity assay for MMP-1

Activity levels of secreted MMP-1 from conditioned media of HUVEC were evaluated using a Biotrak Activity Assay (Amersham Biosciences) as per manufacturer's instructions. Samples consisted of collected cell culture media 48 hours after adenoviral infection of cells. Collected culture media was diluted 1:10 in 1x calibrator diluent before MMP-1 determination. All samples were run in triplicates and compared to a standard curve performed according to the manufacturers' instructions.

Western Blots

Protein expression in HUVEC was evaluated by immunoblotting using a human β -catenin antibody from mouse (BD Transduction Laboratories, CA), human MMP-1 antibody from mouse (R&D Systems Inc, MN) or an α -tubulin antibody (Sigma-Aldrich Life Sciences, MO) when corresponding. For the evaluation of cytosolic β -catenin levels, HUVECs were infected with adenoviruses encoding for Wnt-1, Wnt-5a or control gene (LacZ) as described above and seeded on 10 cm diameter dishes. Cytosols were isolated 48 hrs later by first washing the cells with ice cold PBS three times and then adding 750 μ l of physiological buffer (10 mM TRIS-HCl pH=7.4, 140 mM NaCl, 5 mM EDTA, 2 mM DTT, 0.5 mM PMSF, 0.2 μ g/mL aprotinin, 0.1 mg/mL leupeptin) to each dish. Cells were scraped with a rubber policeman and lysed in a chilled Dounce homogeneizer. Lysates were cleared from unbroken cells and nuclei by centrifugation at 500g for 10 min and the supernatants were then centrifuged at approx. 100,000g for 90 min. Cytosolic fractions were frozen at -20°C until the protein content was evaluated with a human β -catenin antibody. For the Western blot of MMP-1 and α -tubulin, lysates were prepared as indicated by the manufacturer of the corresponding antibody. Proteins were visualized by Enhanced Chemiluminescence (Amersham) in conjunction with goat anti-mouse IgG-HRP.

Appendix 3

"Wnt/ β -catenin Signaling Induces Proliferation, Survival and Interleukin-8 in Human Endothelial Cells"
Angiogenesis, 2005 (in press).

T. Néstor H. Masckauchán, Carrie S. Shawber, Yasuhiro Funahashi, Chi-Ming Li and Jan Kitajewski.

Wnts are secreted signaling proteins able to control diverse biological processes such as cell differentiation and proliferation. Many Wnts act through a canonical, β -catenin signaling pathway. Here, we report that Wnt receptors and transcriptional effectors are expressed in primary human endothelial cells and that Wnt/ β -catenin signaling promotes angiogenesis. Human umbilical vein and microvascular endothelial cells express Wnt receptors, Frizzled-4, -5, -6, and β -catenin-associated transcription factors, Tcf-1, -3, -4 and Lef-1. In endothelial cells, ectopic expression of Wnt-1 stabilized cytosolic β -catenin, demonstrating activation of the Wnt/ β -catenin canonical signaling pathway. Expression of Wnt-1 or a stabilized and active form of β -catenin, β -cateninS37A, promoted endothelial cell proliferation. Proliferation induced by Wnt/ β -catenin signaling was optimal in the presence of bFGF. β -cateninS37A expression in endothelial cells promoted survival after growth factor deprivation. Using matrigel assays, Wnt-1 or β -cateninS37A expression promoted the formation of capillary-like networks. To help define the effectors of Wnt angiogenic function, microarray analysis was used to compare endothelial cells expressing Wnt-1 to control cells. Interleukin-8 (IL-8), a known angiogenic factor, was identified as a transcriptional target of Wnt/ β -catenin signaling in endothelial cells. Expression of either Wnt-1 or β -cateninS37A induced IL-8 transcripts and secreted protein. We thus conclude that Wnt/ β -catenin signaling promotes angiogenesis possibly via the induction of known angiogenic regulators such as IL-8.

Appendix 4

"Alternative Wnt Signaling Pathways in human primary endothelial cells"

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Era of Hope Meeting – Philadelphia, PA – June 8th to 11th, 2005

The Wnt signal transduction pathway has been implicated in mammary tumorigenesis in different species as well as in human breast cancer cell lines. Wnts are known to activate several different signaling pathways through the same receptor and elicit unique cellular responses. Besides the so called canonical Wnt/ β -catenin pathway, other branches of Wnt signaling has been described, such as activation of JNK or Rho GTPases. The present report shows data obtained in the setup of assays to determine the activity of these signaling pathways, as well the biological effects of non-canonical Wnt signaling. In RhoA assays, a canonical Wnt (Wnt-1) and two non-canonical Wnts (Wnt-5A and Wnt-6) were able to stimulate RhoA activity in 293-derived cells (not shown). In the same cells, Wnt-1 also showed a moderate upregulation of Rac1 GTPase. Additionally, the role of non-canonical Wnt signaling in angiogenesis was explored. Human umbilical vein endothelial cells (HUVEC) were infected with adenovirus encoding for Wnt-5A or control gene LacZ. Wnt-5A overexpressing cells showed a higher rate of proliferation and cell survival (Fig. 1 A-B), as well as upregulation of matrix metalloproteinase-1 (MMP-1) both at a transcriptional level by RT-PCR and protein level by Western blot and an ELISA assay (Fig. 2 A-C). As Wnt proteins have shown to be important in both mammary gland normal development and breast tumorigenesis, contributions to further elucidation of the signaling events beyond Wnt activation in breast cancer cells and in angiogenesis are crucial in the design of future strategies to fight breast cancer.