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13. Abstract *(Maximum 200 Words) (abstract should contain no proprietary or confidential information)*
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 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 as the construction of plasmids for the project. A mouse mammary cell line was prepared ectopically expressing Wnt-1. 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 . In the same cells, Wnt-1 also showed a moderate upregulation of Rac1 GTPase.

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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 canonical β-catenin/Tcf/Lef, as well as the activation of small Rho GTPases (guanosine-triphosphatases) and JNK (c-Jun NH_2-terminal kinase) signaling. This Wnt/Frizzled pathway appears to employ Dvl to stimulate RhoA and Rac/JNK activities and is likely mediated by independent Dvl-RhoA and Dvl-Rac complexes induced upon Wnt signaling (Habas et al., 2001). There are clear evidences of a role of canonical Wnt signaling in breast cancer (Howe et al., 2004; Hatsell et al., 2003), and in light that a canonical Wnt like Wnt-1 can also upregulate non canonical signaling, its role in breast cancer cells needs to be further investigated.

Report Body

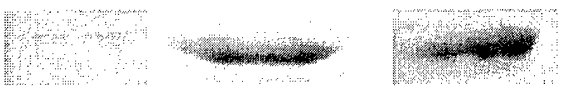
As expressed in the Statement of Work, task 1, items a and b were performed while item c is currently under execution. Adenoviral stocks of viruses encoding several Wnts were tested for expression in human cells (figure 1).

Wnt-1 Wnt-5A Wnt-6 Wnt-7A LacZ

Figure 1. Ectopic expression of Wnts. HUVEC infected with adenoviruses encoding for different Wnt-HA proteins at 30 MOI (multiplicity of infection). Cells were collected 48 hours after infection and lysates were prepared and electrophoresed as described by Shimizu et al. (1997) using an anti-HA antibody for detection followed by a HRP-tagged secondary antibody incubation and ECL chemiluminescent assay.

Also, mammary cell (C57MG) lines encoding for either Wnt-1 or empty vector were obtained by retroviral infection and hygromycin selection. Mammalian expression vectors were constructed/prepared containing encoding sequences for several Rho family GTPases: dominant negative forms $RhoA^{N19}$, $Rac1^{N17}$ and $Cdc42^{N17}$, which preferentially bind GDP rather than GTP, constitutively active mutants $RhoA^{V14}$, $Rac1^{V12}$ and $Cdc42^{V12}$, as well as Rac1 (Wilt type), JNK wild type, myc-Dbl.

RhoA, Rac1 and Cdc42 assays were set up in Bosc cells. For this purpose, bacterial expression plasmids encoding for the fusion proteins GST-RBD (rhotekin binding domain) and GST-PBD (pak binding domain). Fusion proteins were successfully obtained and bound to Sepharose-glutathione beads (figure 2) which were used in GTPase assays as described in *Methods* in the proposal. Only active GTPases will be able to bind RBD- or PDB-GST proteins bound to Sepharose-glutathione, and therefore detected by Western blot of boiled beads. Commercial antibodies anti-RhoA, Rac1, Cdc42 and phospho-JNK were also obtained and tested. Also, cJun-GST beads were prepared (figure 2) and used to evaluate JNK activity in Bosc cells.

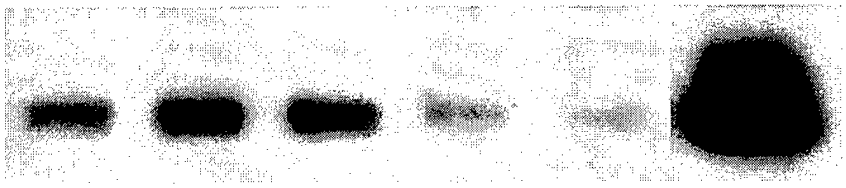


RBD-GST PBD-GST c-Jun-GST

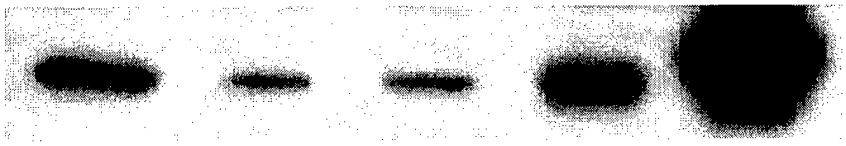
Figure 2. Fusion proteins were prepared and bound to Glutathione Sepharose 4B beads as described under *Methods* in the proposal. Boiled beads were electrophoresed in SDS-PAGE gels and gels were then stained with Coomasie Blue and photographed.

In Bosc cells, a 293-derived cell line, a canonical Wnt (Wnt-1) and two non canonical Wnts (Wnts 5A and 6) were able to stimulate RhoA activity (figure 3A). In the same cells, Wnt-1 also induced a moderate upregulation of Rac1 activity (figure 3B). Wnt-1 induction of RhoA activity is in line with reported observations that Wnt-1 conditioned culture media can induce activation of RhoA and Rac1 but not Cdc42 in 293 cells (Habas et al., 2001). Activated JNK (observed as two

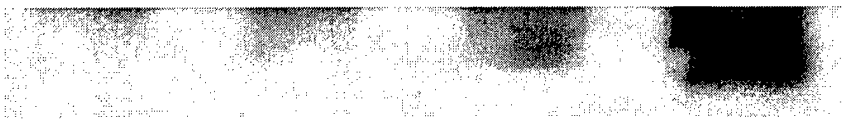
bands of 46 and 54 kDa) was not detected after ectopic overexpression of any of the tested Wnts in the experimental conditions used (figure 4).



Wnt-1 Wnt-5A Wnt-6 Wnt-7A LacZ Dbl (A)

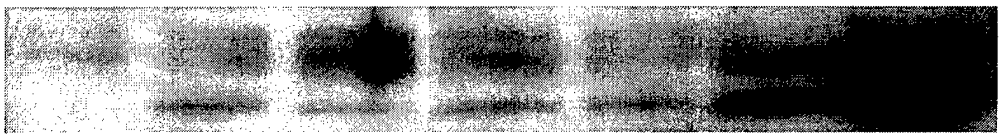


Wnt-1 Wnt-5A LacZ Dbl Rac1V12 (B)



Wnt-1 Wnt-5A LacZ Dbl (C)

Figure 3. (A) Small GTPases assays in Bosc cells. Cells were transfected with plasmids using the calcium phosphate protocol. After 48 hours, cells were harvested and lysates were prepared as described in the *Methods* section of the proposal. Lysate samples were bound to either RBD-GST- (RhoA) or PBD-GST-bound Sepharose 4B beads and analysed by SDS-PAGE followed by Western blot using specific antibodies for each GTPase. The small GTPases analysed were detected as single bands of 21-24 kDa. (A) RhoA assay and (C) Cdc42 assay. Transfections with plasmids encoding for Wnts, negative (LacZ) or positive (Dbl protein) controls. (B) Rac1 assay. Transfections with plasmids encoding for Wnts, negative (LacZ), positive (Dbl) controls or a constitutively active form of Rac1 (Rac1V12).



Wnt-1 Wnt-5A Wnt-6 Wnt-7A LacZ UV light Anisomycin

Figure 4. JNK assay in Bosc cells. Cells were transfected with plasmids using the calcium phosphate protocol. After 48 hours, cells were harvested and lysates were prepared as described in the *Methods* section of the proposal. Lysate samples were bound to c-Jun-GST-bound Sepharose 4B beads and analysed by SDS-PAGE followed by Western blot with an anti-phospho-JNK antibody as primary. Both UV light exposure and anisomycin activation were tested as positive controls of JNK activation.

Key research accomplishments/Reportable outcomes/Conclusions

- Reagents were prepared and plasmids were obtained for the project.
- Techniques/methods were tested and set up.
- Work is currently in progress using mammary gland cell lines.
- Additionally, the PI participated in laboratory research work related to Wnt signaling in two other systems, including a project on Wnt-1 canonical signaling in Rat-1 fibroblasts and Wnt signaling in endothelial cells. Briefly, ectopic expression of β-catenin-S37A was shown not to be enough to promote growth of Rat-1 cells in serum-free conditions and was only partially able to promote growth post-confluence, when compared to that induced by Wnt-1 expression. As

ectopic expression of β-catenin-S37A could only partially mimic Wnt-1 effects on Rat-1 cells, it can be concluded that Wnt-1 signaling elicits biochemical events that act in addition to beta-catenin/Tcf signaling to promote cell growth. This work resulted in a coauthorship in a recent publication (Young et al.; October, 2003) in *Differentiation* (see abstract in Appendix 1). Also, completion of work performed on both canonical and non-canonical Wnt signaling in human primary endothelial cells resulted in a poster presentation at the Wnt Meeting 2004, University of Michigan, Ann Arbor, MI (Masckauchan et al.; May, 2004) (see abstract in Appendix 2).

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Appendix 1

Differentiation. 2003 Oct;71(8):477-85

Beta-catenin/Tcf activation partially mimics the transforming activity of Wnt-1 in Rat-1 fibroblasts.

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We have previously demonstrated that Wnt-1 induction of cytosolic beta-catenin and Tcf/Lef transcriptional activation correlate with enhanced proliferation, survival, and post-confluent growth in Rat-1 fibroblasts. To examine whether beta-catenin mediates the biological responses to Wnt-1 in this context, we characterized Rat-1 clonal cell lines expressing different levels of a mutant, stabilized beta-catenin (beta-cateninS37A). Clonal lines exhibit elevated cytosolic and nuclear beta-cateninS37A and Tcf transcriptional activation, comparable to that elicited by Wnt-1 expression. However, expression of beta-cateninS37A does not promote growth of Rat-1 cells in serum-free conditions and only partially promotes growth post-confluence, when compared to that induced by Wnt-1 expression. As ectopic expression of beta-cateninS37A only partially mimics Wnt-1 effects on Rat-1 cells, we conclude that Wnt-1 signaling elicits biochemical events that act in addition to beta-catenin/Tcf signaling to promote cell growth.

Appendix 2

Canonical and non-canonical Wnt signaling can induce proliferation, cell survival and upregulation of IL-8 or MMP1 in endothelial cells

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Angiogenesis, the formation of new vessels by sprouting from pre-existing ones, is a critical step in embryonic development, pregnancy and placentation, as well as wound healing in tissue repair. The focus of our research are the in vitro and in vivo biological effects of different Wnt components in the vasculature and the Wnt signaling branches underlying behind these effects. We first tested the differential expression of several Wnt family genes in primary endothelial cells in vitro. We have found that, in different amounts, HUVEC (human umbilical vein endothelial cells) endogenously expressed Wnt members 5A and 10B, HUAEC (human umbilical artery endothelial cells) expressed Wnt member 5B and HMVEC (human microvascular endothelial cells) expressed Wnt members 5A, 5B, 7A, 7B and 10B. All these primary endothelial cells lines expressed Frizzled members 4, 5 and 6. Also, we were able to detect that both canonical and non-canonical Wnts can regulate proliferation of endothelial cells in vitro. We tested endothelial cells infected with adenoviruses encoding for Wnt-5A as well as beta-catenin-S37A (a beta-catenin mutant with a longer half life) and incubated in presence of basal medium added with FGF. Cells overexpressing both Wnt-5A and mutant beta-catenin were able to proliferate faster. They also showed an enhanced survival when they were cultured at confluency under the same conditions. After the screening of Wnt adenovirus-infected endothelial cells with a DNA array chip that includes over 10,000 human genes, we focused our attention on interleukin-8 (IL-8) in Wnt-1 adeno-infected cells and matrix metalloproteinase-1 (MMP1) in Wnt-5A adeno-infected cells. We were able to validate the regulation of these genes, being IL-8 upregulated 6.7 fold by canonical Wnt signaling while MMP-1 was upregulated 2 fold by Wnt-5A.