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TABLE OF CONTENTS:

ABSTRACT

Cadherin-11 is unique amongst cadherins in that it is exists as two alternatively spliced forms that are expressed together in the same cell. In year one of this grant we showed that the presence of the cadherin-11 splice variant promotes invasion of cadherin-11 positive breast cancer cells, perhaps by promoting cell-ECM interactions. In year two of the grant we showed that inhibition of cadherin-11 function using proprietary small molecule cadherin-11 inhibitors influences the ability of cadherin-11-expressing cells to invade in vitro. In year three of the grant we continued our work on the small molecule inhibitors of cadherin-11 and characterized cells lines expressing cadherin-11 siRNA. Our work also showed effects of cadherin-11 blockade on the expression of the angiogenic molecules VEGF-A and VEGF-D. In year three and four we investigated the regulation of cadherin-11 expression in breast cancer cells and showed marked effects of cell density and of wnt and TGF-beta signaling. Remarkably, this regulation was mediated in part at the level of mRNA degradation. Finally, a collaborative study showed that cadherin-11 was increased in human breast cancer cells that were selected for their ability to metastasize to bone.

Introduction:

Cadherins play a critical role in establishing adherens-type junctions by mediating Ca^{2+}-dependent cell-cell adhesion. Specific homophilic cell-cell adhesion plays a key role in tissue and organ development during embryogenesis and in maintenance of normal tissue structure in adult organisms (Ivanov *et al.*, 2001). Cadherin-based cell-cell adhesion is involved in early embryonic morphogenesis. For example, mice lacking E-cadherin exhibited early embryonic lethality (Riethmacher *et al.*, 1995)

Cadherins are transmembrane glycoproteins that participate in the formation of homophilic cell-cell adhesion complexes. An extracellular protein domain consisting of a varying number of highly homologous cadherin domains structurally characterizes cadherins. Classical cadherins contain five extracellular cadherin domains harboring calcium-binding sites that are located between neighboring extracellular repeats (Ivanov *et al.*, 2001). It was found that stability of the cadherin protein and subsequent cadherin-mediated cell-cell adhesion was dependent on Ca^{2+} binding to the extracellular domain of the protein.

The intracellular protein domain of cadherins serves to bind cytoplasmic proteins including $p120^{ctn}$, β-catenin and plakoglobin. Hereby, β-catenin acts as a linker between the cadherin domain and α-catenin. Further, the catenins mediate the interaction between the cadherin protein and the cytoskeleton including actin and vinculin (Yagi *et al.*, 2000).

Cadherin-11 was first cloned independently from a mouse osteoblastic cell line MC3T3-E1 and mesodermally derived tissues (Okazaki *et al.*, 1994; Hoffman *et al.*, 1995). During development cadherin-11 was expressed predominantly in mesenchymal components of organs. In contrast, E-cadherin was found exclusively in epithelial structures, as was N-cadherin. Cadherin-11 appeared to be down-regulated upon differentiation of tissues (Hoffman *et al.*, 1995).

A cadherin-11 isoform, termed cadherin-11 variant was found to encode a truncated protein with an altered cytoplasmic domain. The cytoplasmic protein domain resembled no part of the cytoplasmic domain in other cadherins. Gene analysis revealed an insertion of 179 bp in the transmembrane domain hence generating an alternatively spliced form (Kawaguchi *et al.*, 1999). The protein size of the cadherin-11 variant form is ~85 kd in comparison to full-length cadherin-11 with a size of ~120 kd. The cad-11 variant form is unable to associate with β-catenin as the cytoplasmic domain does not possess a catenin-binding site. Cell aggregation studies suggested the variant form to enhance adhesion kinetics when L cells were transfected with cadherin-11 or cadherin-11 in combination with the variant form. In addition, it was suggested that cadherin-11 variant enhances the association between cadherin-11 and the cytoskeleton via β-catenin thereby stabilizing the full-length cadherin-11 (Kawaguchi *et al.*, 1999). Distribution of variant mRNA in tissues and cells appeared the same as cadherin-11 but expression levels were lower.

Previously, loss of expression or function of the epithelial cell-cell adhesion molecule E-cadherin was found to be associated with a loss of epithelial phenotype and with a gain of invasiveness in a number of cancers including breast cancer (Pishvaian *et al.*, 1999). In contrast expression of cadherin-11 and its variant form were found to be expressed in the most invasive breast cancer cell lines but were not detected in non-invasive cell lines (Pishvaian *et al.*, 1999). Further, it was proposed that expression of the cadherin-11 splice variant promotes invasion of cadherin-11-positive breast cancer cells (Feltes *et al.*, 2002). Hence cadherin-11 expression was suggested to be correlated with the invasive phenotype in cancer cells and could potentially serve as a predictor of the invasive and metastatic phenotype.

The vascular endothelial growth factors have been implicated in the progress of angiogenesis *in vivo* and *in vitro* and lymphangiogenesis (Marconcini *et al.*, 1999). The family of vascular endothelial growth factors (VEGFs) encompasses six proteins including VEGF-A, -B, -C, -D, placenta growth factor (PlGF) and the viral homologues, collectively called VEGF-E (Karkkainen *et al.*, 2000). VEGFs all possess a VEGF homology domain that spans approximately 100 amino acids and is characterized by the precise spacing of 8 cysteine residues. VEGFs are specific ligands that bind to VEGF-receptors (VEGFR) in order to initiate molecular processes involved in angiogenesis and lymphangiogenesis. PlGF and VEGF-B bind to VEGFR-1 whereas VEGF-A interacts with both VEGFR-1 and VEGFR-2. VEGF-C and VEGF-D bind VEGFR-2 and VEGFR-3 (Achen *et al.*, 1998; Karkkainen *et al.*, 2000).

Expression studies of VEGF family members in breast cancer cell lines found all VEGFs to be expressed in the seven cell lines analyzed, despite at varying levels (Kurebayashi *et al.*, 1999). Further investigation into the expression levels of VEGFs in human breast tumors revealed VEGF-A and VEGF-B to be expressed in both node-positive and - negative tumors while VEGF-C expression was only detected in node-positive tumors. VEGF-D was detected in inflammatory breast cancer and a tumor that had developed an inflammatory skin metastasis (Kurebayashi *et al.*, 1999). An additional study concerned the prognostic value of the splice variants of VEGF-A with respect to expression levels in breast and ovarian cancer (Stimpfl *et al.*, 2002). Results showed $VEGF^{121}$ and $VEGF^{165}$ to be the most dominantly expressed variants of VEGF-A in all tumor samples and cell lines analyzed whereas $VEGF^{145}$ was weakly or not expressed in breast and ovarian cancers. No correlation between VEGF-A splice variant expression in tumors and histological type, differentiation grade, tumor size and nodal status from cancer patients was found. Therefore, the study concluded that no correlation between the invasive capacity of breast cancer cell lines and VEGF-A isoforms was apparent (Stimpfl *et al.*, 2002).

Finally, studies found expression of VEGF-D to be induced by cadherin-11 mediated cell-cell interaction in fibroblasts (Orlandini *et al.*, 2001). It was demonstrated that in non-interacting cell expression of VEGF-D was low while VEGF-D expression was high in contacting cells. Calcium deprivation and its associated loss of cadherin-11 from the cell surfaces also resulted in subsequent loss of VEGF-D transcript. Inhibition of cadherin-11 using specific anti-sense RNA constructs resulted in a loss of VEGF-D mRNA in confluent BALB/c fibroblasts. In contrast, increased expression of cadherin-11 in NIH3T3 cells led to induction of VEGF-D expression (Orlandini et al., 2001).

In conclusion, the above reports suggest that cadherin-11 might be able to facilitate breast cancer cell invasion resulting in angiogenesis and lymphangiogenesis and therefore examination of the mechanism by which cadherin-11 promotes invasion and the relationship of cadherin-11 to VEGF and cell invasion is essential.

Body:

Results

LiCl and TGF-beta 1 treatments attenuate density dependent regulation of cadherin-11

As demonstrated in previous reports, increased cell density, increases cadherin-11 expression whereas LiCl and TGF-beta 1 treatment decrease cadherin-11 expression. In order to determine whether LiCl or TGF-beta 1 could abrogate the cell density increase in cadherin-11, cadherin-11 expressing cells grown at varying densities were treated with either LiCl or TGF-beta 1. Treatment with either LiCl or TGF-beta 1 decreased both cadherin-11 RNA and protein expression (Figure 1). LiCl dramatically reduced cadherin-11 RNA (Figure 1A) to lower than basal expression, which is reflected at the protein level (Figure 1C). TGF-beta 1 has a less marked effect but also attenuates the density-dependent effect on cadherin-11

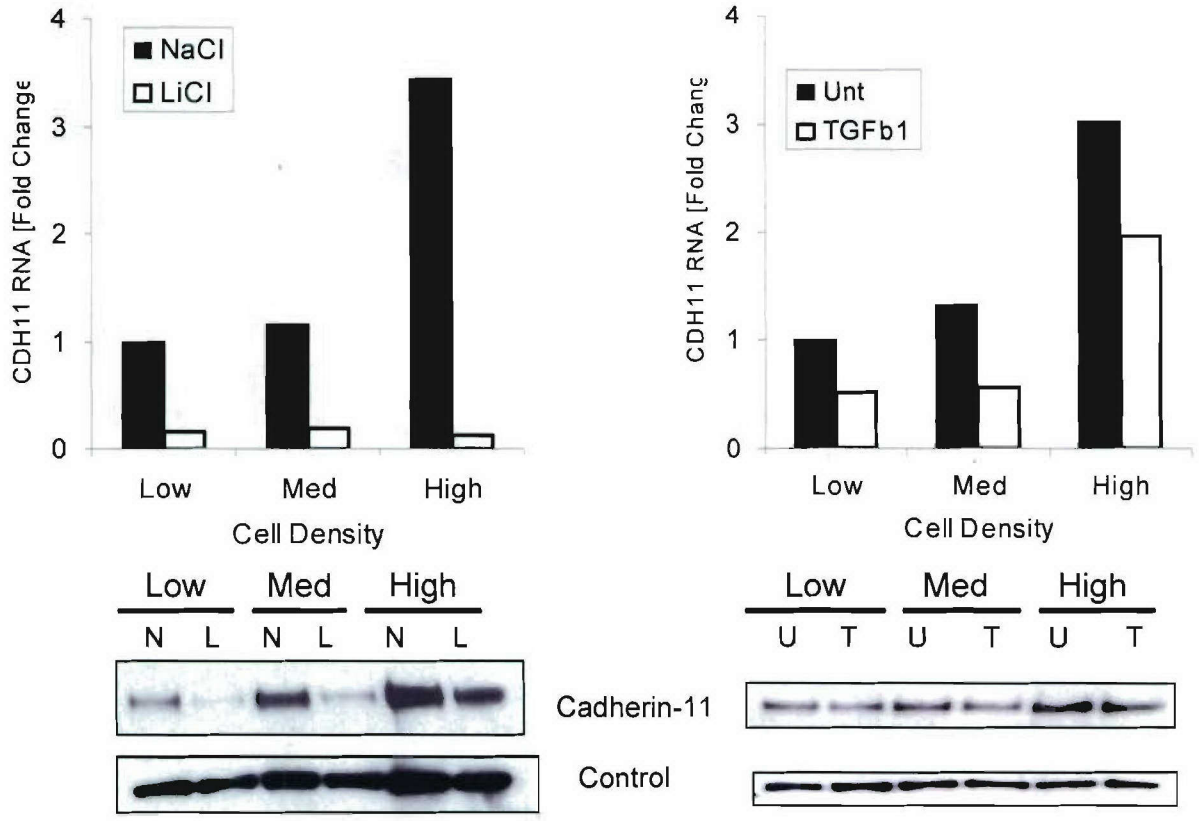

Figure 1: LiCl and TGF-beta 1 treatments attenuate density dependent regulation of cadherin-11

expression levels (Figures 1B and 1D). Because the best known mechanism of affecting target gene expression of both beta-catenin and Smad proteins is through transcription, the effect of LiCl on cadherin-11 transcription was investigated.

The effect of LiCl appears to have a transcriptional as well as a post-transcriptional component. To determine whether the observed effects of LiCl on cadherin-11 expression are transcriptional in nature, the effect of actinomycin D, a transcriptional inhibitor, was investigated. As expected, cells treated with actinomycin D alone showed a decrease in cadherin-11 RNA expression with time. LiCl treatment also reduced cadherin-11 levels after 6 hours, where as actinomycin D treatment alone required twice as much

time to result in the same level of inhibition (Figure 2), implying that LiCl is not regulating cadherin-11 through a transcriptional means, but possibly by a post-transcriptional mechanism. The most straightforward method of post-transcriptional regulation is by disruption of RNA stability, which would decrease both RNA and protein levels. It is known that the sequence AUUUA in a 3'-UTR is critical for the RNA stability of a gene. Indeed there are multiple AUUUA sequences in the 3'-UTR of human cadherin-11 gene. There is a report showing that increased cytoplasmic beta-catenin levels resulted in destabilization of VEGF-D RNA via its 3'-UTR (Orlandini et al., 2003). Therefore, it is also possible that LiCl induced beta-catenin affects the stability of cadherin-11 RNA also via the 3'-UTR, resulting in decreased cadherin-11 RNA and protein.

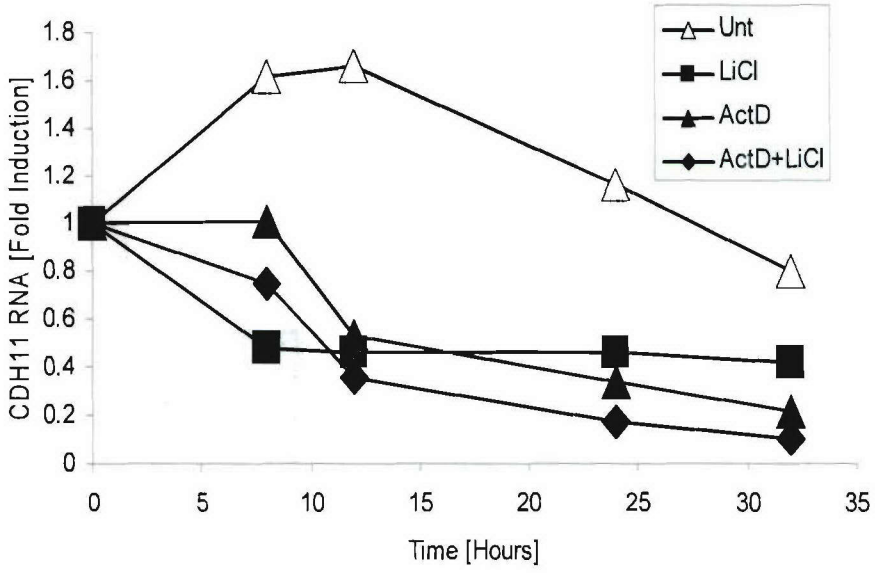

Figure 2: LiCl attenuation of cadherin 11 mRNA has a post-transcriptional component.

Cadherin-11 is elevated in MDA-231 cells that metastasize to bone:

In collaboration with Dr. Rik Thompson at the University of Melbourne, Australia we have found that MDA-231 cells specifically selected for bone metastasis express considerably more cadherin-11 than parental cells.

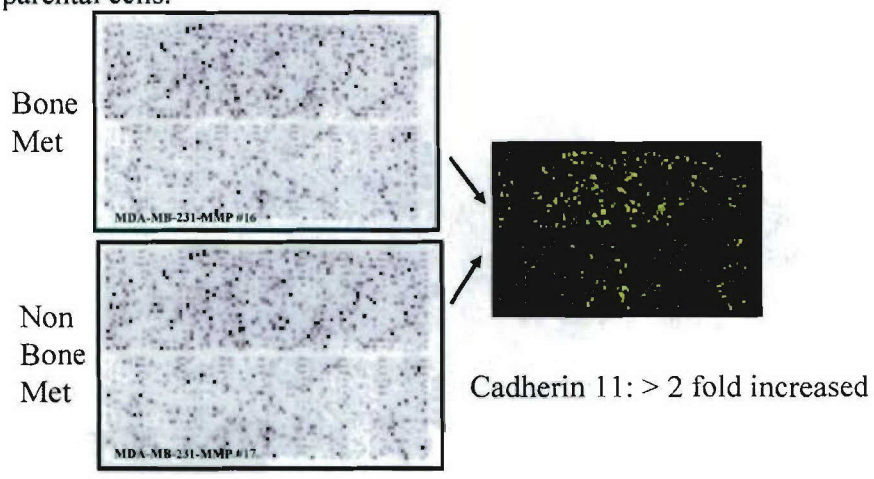

Figure 3. ***Microarray analysis indicates that cadherin-11 is upregulated specifically in cells that have metastasized to bone. Cells derived in the Thompson laboratory.***

This was true in cells generated in the Thompson laboratory (figure 3) and in cells generated by the Yoneda laboratory (Figure 4) and strongly indicates that expression of cadherin-11 and/or its variant form does indeed predispose to bone metastasis.

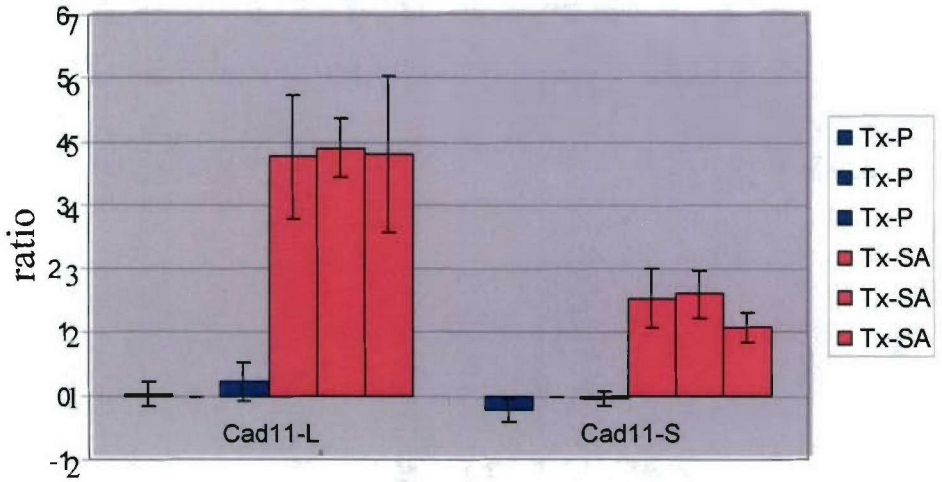

Figure 4. ***qPCR analysis indicates that cadherin-11 is upregulated specifically in cells that have metastasized to bone. Cells derived in the Yoneda laboratory (Tx-SA) compared to parental cells (Tx-P).*** **Cad11-L (variant), Cad11-S (wild type cadherin-11)**

Key Research Accomplishments:

Year 1:
1. Demonstration that cadherin-11 can either promote cell-cell adhesion or promote invasion depending upon the concommitant expression of the cadherin-11 variant.
2. Generation of a cadherin-11 variant antibody:
3. Generation of cadherin-11 ribozyme expressing cell lines:

Year 2:
1. Demonstration that cadherin-11 blocking small molecules can inhibit the invasion of cadherin-11-expressing cells.
2. Generation of cadherin-11 siRNA-expressing cell lines:

Year 3:
1. Demonstration that cadherin-11 blocking small molecules can regulate VEGF expression by cadherin-11-expressing cells.
2. Cells that express cadherin-11 siRNA make very low levels of cadherin-11 but normal levels of cadherin-11 variant and are more invasive than control cells.
3. Cadherin-11 expression is repressed by cell density, beta-catenin signaling and by TGF-beta.

Year 4:
1.Wnt signaling and beta catenin regulate cadherin-11 expression in part by affecting RNA stability. TGF-beta regulates cadherin-11 expression at the transcriptional level.
2. Two independently selected human breast cancer cell lines that preferentially home to bone express 2-7 fold more cadherin-11 (collaboration with Rik Thompson-University of Melbourne, Australia)

Reportable Outcomes:

Publications:
Feltes, C, Kudo, A. Blaschuk, O. and Byers, S. An alternatively spliced cadherin-11 regulates human breast cancer cell invasion. (2002) Cancer Res 62:6688-6697
Hampel, C, Blaschuk, O, Rowlands, T. and Byers, S. Small molecule antagonists of cadherin-11 function alter breast cancer cell invasion and VEGF expression. In preparation
Kawczenski, A, Feltes, CM, and Byers SW. Post-transcriptional regulation of Cadherin-11 expression by wnt/catenin signaling. In preparation

Presentations:
Mesenchymal cadherins and breast cancer. Invited speaker at the Hormonal Carcinogenesis Gordon Conference. New Hampshire. July 2001.
Cadherin-11 and carcinoma cell invasion and metastasis. Urology Department, McGill University, October, 2002

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Jaime Guidry
Becky Hoxter

Conclusions:

Previously, loss of expression or function of the epithelial cell-cell adhesion molecule E-cadherin was found to be associated with a loss of epithelial phenotype and with a gain of invasiveness in a number of cancers including breast cancer (Pishvaian *et al.*, 1999). In contrast expression of cadherin-11 and its variant form were found to be expressed in the most invasive breast cancer cell lines but were not detected in non-invasive cell lines (Pishvaian *et al.*, 1999). In work supported by DAMD 17-01-1-0245 we showed that expression of the cadherin-11 splice variant promotes invasion of cadherin-11-positive breast cancer cells (Feltes *et al.*, 2002). Hence cadherin-11 expression was suggested to be correlated with the invasive phenotype in cancer cells and could potentially serve as a predictor of the invasive and metastatic phenotype. Our data also show that a particular class of inhibitors designed to block the ability of cadherin-11 to interact with the extracellular matrix does indeed affect cell invasion and the ability of cells to express VEGFs. Other molecules designed to disrupt the cell-cell adhesive function of cadherin-11 did not affect cell invasion or VEGF expression. The demonstration that small molecule inhibitors can effectively block this important function of cadherin-11 bodes well for the development of drugs that can inhibit the ability of cadherin-11 expressing cells to stimulate blood vessel growth and metastasize. In other studies we generated cell lines expressing siRNA directed at both cadherin-11 and cadherin-11 variant. These data strongly indicate that inhibition of the ability of cadherin-11 to interact with the ECM blocks cell invasion. Experiments in progress are directly investigating the ability of cadherin-11 deficient cells to metastasize to bone. Other experiments show that at least a component of wnt/catenin regulation of cadherin-11 is mediated at the post-transcriptional level. F inally, in collaboration with Dr. Rik Thompson at the University of Melbourne, Australia we have found that MDA-231 cells specifically selected for bone metastasis express considerably more cadherin-11 than parental cells. This was true in cells generated in the Thompson laboratory and in cells generated by the Yoneda laboratory and strongly indicates that expression of cadherin-11 and/or its variant form does indeed predispose to bone metastasis.

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