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14. ABSTRACT
Increased genomic instability arising from centrosomal amplification has been proposed to be an important factor causing development of traits associated with highly malignant ovarian tumors, including multidrug resistance and increased tendency to metastasis. This proposal addresses the hypothesized interaction between the Cas proteins (HEF1 and p130Cas), Aurora A (AurA) and Ajuba as being likely to contribute to genomic instability and metastatic properties of ovarian tumors. Our work during the no cost extension has begun investigating the association between HEF1 overexpression and AurA activation in primary ovarian tumors, with data suggesting a potential correlation. We have also used mouse models to directly assess the effect of modulating HEF1 on cancer progression, and have data suggesting the absence of HEF1 also predisposes cancers to abnormal mitoses, centrosomal defects, and genomic instability. In the past three years, elevation of HEF1 has been established as important for metastasis and/or invasion in lung cancer, melanoma, and glioblastoma, while reduction of HEF1 has been suggested as relevant inmetastatic breast cancers; our ongoing work will establish the role of HEF1 in ovarian cancer.

15. SUBJECT TERMS
HEF1, NEDD9, Aurora-A, centrosome, genomic instability

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INTRODUCTION: The goal of this proposal is to better understand factors leading to ovarian cancer development, with the intent of improving diagnosis and treatment for ovarian cancer. Our preliminary data defined the HEF1 member of the Cas protein family as a regulator of centrosomal dynamics and genomic instability through control of the Aurora A (AurA) kinase. The goal of the proposal was to explore HEF1 and p130Cas protein status as a contributing factor to early onset of ovarian cancers, and use this information to assess the value of combining targeted small molecule therapeutics for ovarian cancer therapy. Three Aims were proposed to address this objective. In *aim 1*, we proposed to examine tumor samples to determine if HEF1 and/or p130Cas expression, AurA activation, and centrosomal amplification are linked, and whether Cas protein upregulation is associated with a poor prognosis. In *aim 2*, we proposed to examine the mechanism by which Cas proteins activate AurA, and determine if drug-mediated inactivation of AurA inhibits Cas promotion of aneuploidy. In *Aim 3*, we proposed to use further drug and depletion experiments to determine if centrosome amplification and enhanced cellular metastasis are linked, and dependent on Cas/integrin signaling, or whether these are separable properties; and to evaluate combination of AurA- and integrin- directed therapies.

BODY: During the previous reporting period (11/06-11/07), we have almost completed work on Aims 2 and 3, resulting in a research publication in Cell and reviews. An additional research publication is in preparation. Because of our focus on the latter two aims, we did not extensively advance our work on Aim 1, and requested a *one-year, no-cost extension* of this grant to allow us to complete this work.

<u>Aim 1: Determination of whether HEF1 or p130Cas expression, activated AurA, and centrosomal amplification are linked, and whether Cas protein upregulation is associated with a poor prognosis.</u>

1. We used HEF1-directed antibodies developed by our laboratory (monoclonal Ab [mAb] 2G9[1]) to evaluate HEF1 expression in a panel of 5 primary cultures of normal human ovarian surface epithelial (HOSE) cells and 17 human EOC cell lines (**Figure 1**), with each cell line probed in at least two independent experiments. For this work, we established a novel collaboration with Dr. Denise Connolly, an experienced ovarian cancer researcher [2-8] (see proposed Future Directions in conclusion of last report). HEF1 protein expression was abundant in 8/17 (47%) of EOC cell lysates, but only detectable in 1/5 (20%) of HOSE lysates. Comparison of HEF1 signal intensity with that of T288-phospho (activated) Aurora-A showed relatively poor correlation in cell lines (see representative data, **Figure 2**). This result was unexpected, and differs from that seen in other studies in mammary cell lines (beyond the scope of this proposal). We hypothesized that it may be related to changes occurring during the selection of cell lines from primary tumors. Testing this hypothesis required comparison of primary tumors and cell lines, and also performance of pro-active experiments to assess the consequences of mutating HEF1.

2. We next optimized conditions for use of mAb 2G9 for IHC detection of HEF1 on formalin fixed paraffin embedded primary EOCs, normal ovaries and normal reference tissues (**Figure 3**). 2G9 did not significantly stain normal ovarian surface epithelium (**Figure 3A**), but strongly stained a benign metaplastic epithelial inclusion cyst (**Figure 3B)**, a borderline serous cancer (**Figure 3C)** and some **(Figure 3D-E)**, but not all **(Figure 3F)**, invasive serous cancers. Moderate to intense staining was seen in 6/29 (20%) invasive serous cancers. These staining

patterns suggest that overexpression of HEF1 may be a contributing event in ovarian carcinogenesis, and further that alteration in HEF1 protein levels may be an early event. Taken together, these results suggest that overexpression of HEF1 protein is relatively common in human EOC cell lines and primary tumors.

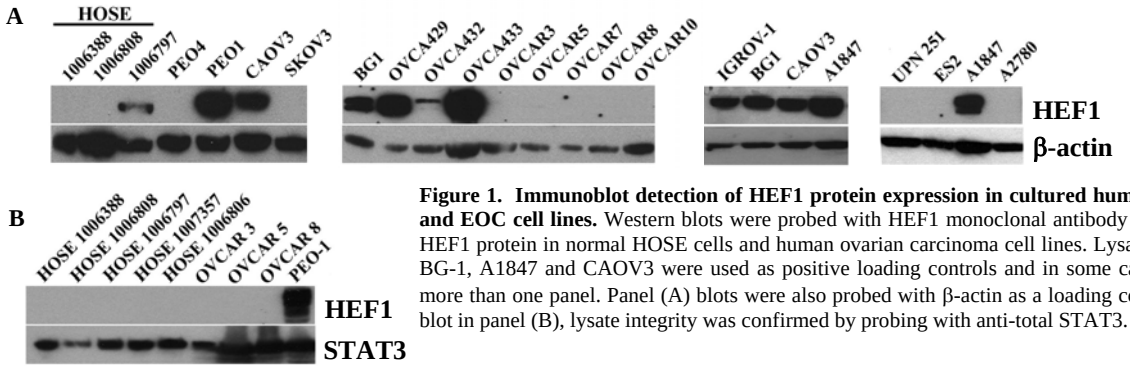

Figure 1. Immunoblot detection of HEF1 protein expression in cultured human OSE cells and EOC cell lines. Western blots were probed with HEF1 monoclonal antibody 2G9 to detect HEF1 protein in normal HOSE cells and human ovarian carcinoma cell lines. Lysates for PEO1, BG-1, A1847 and CAOV3 were used as positive loading controls and in some cases appear in more than one panel. Panel (A) blots were also probed with β-actin as a loading control. For the blot in panel (B), lysate integrity was confirmed by probing with anti-total STAT3.

3. Using tissue microarrays, we next analyzed the co-occurrence of HEF1 expression and T288-phospho (activated) Aurora-A by immunohistochemistry, using the same tissue microarray noted above. For contrast, we also compared expression of phospho-FAK, which is known to correlate with HEF1 expression REFs. Representative data from this work, again performed in conjunction with Dr. Connolly, is shown in **Figure 4**. We successfully optimized use of the phospho-Aurora-A and phospho-FAK antibodies for use in IHC; results indicate that a number of low- and high-grade ovarian tumors are marked by co-overexpression of HEF1 and phospho-Aurora-A. The trend is most notable with low-grade ovarian tumors, which have previously been associated with Aurora-A activation [9-12]; however, we have not yet been able to acquire sufficient numbers of annotated specimens from our Tumor Bank to take the results to statistical significance. We are currently working with Tumor Bank staff to obtain the necessary tissue arrays, and we have been promised the material within the next 60 days. Following extension of these experiments to the larger group of samples, if a statistically significant result is obtained, we will then determine whether centrosomal amplification also correlates with HEF1 overexpression. Use of antibodies to visualize centrosomes by IHC has been well validated, and no difficulties are anticipated with these final experiments.

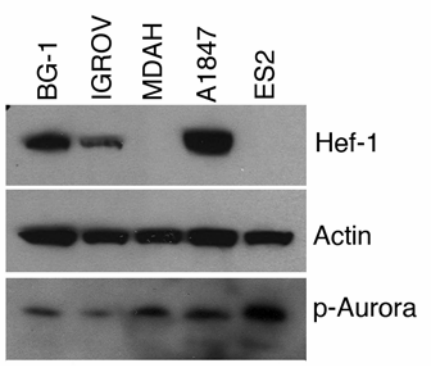

Figure 2. Western blot of cell lines stained with antibodies to HEF1, actin, and T288-phosphorylated (activated) Aurora A. Additional cell lines showed similar results.

4. We have also pursued direct experiments to assess the in vivo correlation of modulation of HEF1 expression, and genomic instability. We have crossed HEF1-/- mice onto two different genetic backgrounds. We note that although most of our work has focused on HEF1 overexpression, loss of HEF1 expression may also have consequences for Aurora-A function and genomic instability: given that HEF1-/- cells have difficulty transiting mitosis due to failed Aurora-A activation, and tend to re-fuse post-anaphase to form binucleate cells that ultimately trigger aneuploidy, as we have published in [1, 13]. One is the very

well-studied MMTV-PyVmT model for mammary tumorigenesis; the second is Dr. Denise Connolly's *MISIIR-TAg* model for ovarian tumorigenesis [14]. Although not ovarian, the advantage of the first, PyVmT model is the rapid and highly penetrant formation of tumors, which allowed us to conveniently obtain and compare cell lines with HEF1+/+ and HEF1-/- genetic backgrounds. Our early data from this work has showed that 1) HEF1-/- tumor-derived cell lines have more abnormal cell cycle profiles than HEF1+/+ derived cell lines, with loss of clear G1/G2 peaks suggestive of aneuploidy (**Figure 5**); and 2) that HEF1-/- cell lines have more amplified centrosomes than HEF1+/+ cell lines, suggestive of failed cytokinesis (**Figure 6**).

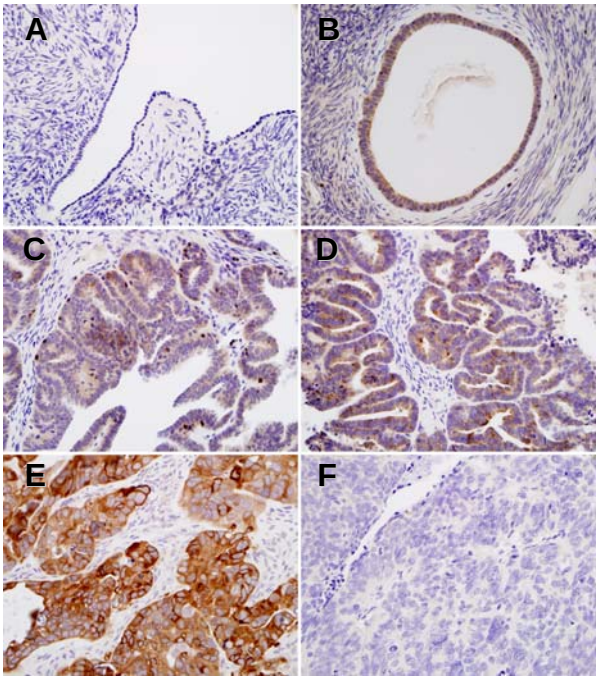

Figure 3. Immunohistochemical detection of HEF1 protein in ovarian epithelium and ovarian tumors. A) Normal ovarian surface epithelium showing little or no HEF1 staining. Strong HEF1 staining in a benign metaplastic ovarian inclusion cyst (B), a borderline serous carcinoma (C), two of three invasive serous carcinomas (D-F). All images are 400x magnified.

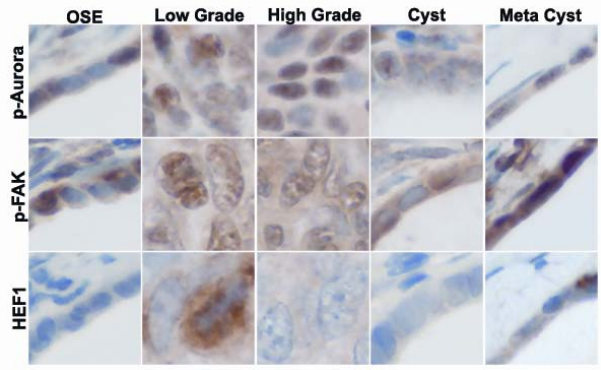

Figure 4. See text for details

5. The Connolly group has had a long-term interest in developing GEM models that spontaneously develop epithelial ovarian cancer (EOC). In their *MISIIR-TAg-DR26* line, the antigen SV40 TAg is expressed under transcriptional control of a portion of the 5' upstream regulatory region of the *MISIIR* gene, and female offspring develop bilateral ovarian carcinomas with 100% penetrance[14]. Ovarian tumors arising in *MISIIR-TAg* transgenic mice exhibit morphology similar to human serous EOCs[14]. Necropsy consistently reveals bilateral ovarian tumors and dissemination of the tumor, with invasion of the omentum, mesentery and parietal and visceral serosa. Since its establishment, this transgenic line has been backcrossed for more than 12 generations to C57Bl/6 and tested with an established panel of markers to ensure genetic purity. Survival data from 77 untreated *MISIIR-TAg* mice showed the mean time to death was 151.6 days (SD = 21.1)[14].

Extensive analyses of mouse ovarian cancer (MOVCAR) cell lines and primary ovarian tumors from *MISIIR-TAg* mice demonstrated numerous features shared with human EOC cell lines and tumors including, but not limited to, overexpression of Cox1 activation of Akt, activation of Stat3 and secretion of VEGF as well as expression of activated Src and MMPs. Importantly, in collaboration with Connolly, we have now shown that Hef1 is overexpressed in most MOVCAR cell lines tested (**Figure 7**), suggesting elevated Hef1 expression is relevant to tumorigenesis in MISIIR-TAg transgenic mice. We have crossed the MISIIR-TAg transgene onto HEF1-/- versus

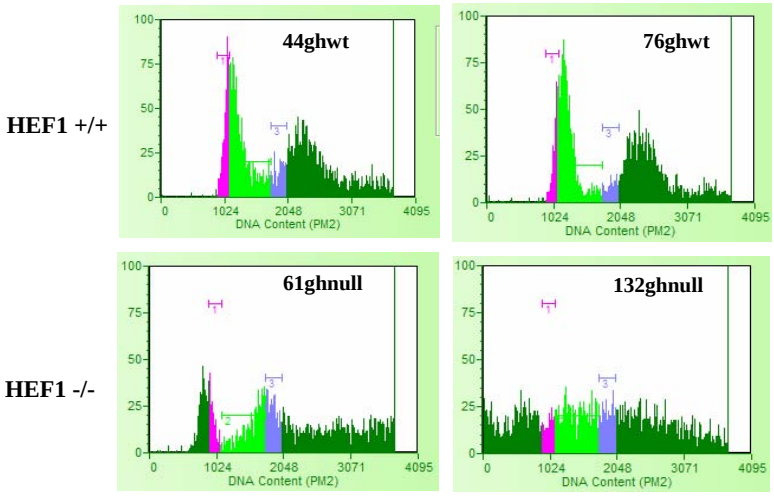

Figure 5. Representative cell cycle profiling of HEF1 wt (+/+) and HEF1 -/- cell lines. HEF1-/- lines tend to have lack of clear peaks for G1/G2, or a large number of >4N cells.

HEF1+/+ genetic backgrounds, and have so far accrued ~ 20 mice of each genotype. These are currently between 30-100 days old: we will use these to assess the consequences of manipulating HEF1 for ovarian tumor development, Aurora-A activation, and genomic instability in early 2009.

<u>Aim 2: Determining the mechanism by which HEF1 and p130Cas proteins activate AurA, and whether inactivation of AurA inhibits Cas promotion of aneuploidy</u>. This Aim was essentially completed at the time of the previous progress report.

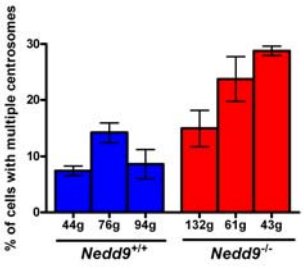

Figure 6. See text for

<u>Aim 3: Determination of whether if centrosome amplification and enhanced cellular metastasis are linked, and dependent on HEF1/integrin signaling, and whether combination of integrin-pathways targeted inhibitors with AurA inhibitors is of clinical merit.</u> This Aim had the following sub-Aims: 1. We will perform structure-function analysis to evaluate whether HEF1 control of cell attachment, and regulation of centrosome-associated functions utilize overlapping or separable domains. 2. We will determine if inhibition of HEF1 signaling by inhibitors targeting the integrin pathway blocks HEF1 ability to induce centrosomal amplification. 3. We will determine whether inhibition of AurA signaling blocks HEF1-dependent cell migration and invasion. 4. We will determine whether combination of AurA- and integrin-targeted inhibitors synergistically blocks both cell migration/invasion, and centrosomal amplification.

As of the last progress report, we had completely addressed sub-Aims 1 and 3, and were planning to address sub-Aims 2 and 4 in 2008. However, due to intensive efforts particularly on Aim 1, and the limited funding available during this period of no-cost extension, we have not been able to undertake the drug studies. We are continuing our close collaboration with the Connolly group, and have together submitted an RO1 to support continued studies of HEF1 in ovarian cancer.

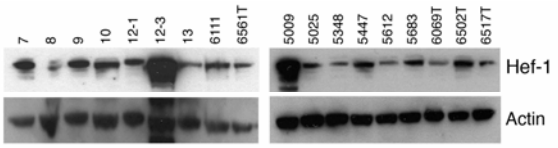

Figure 7. Hef1 protein is frequently overexpressed in MOVCAR cell lines. Hef1 protein was detected in protein lysates of MOVCAR cell lines established from ascites or primary tumors (T) by IB using α-Hef1 Ab 2G9. β-Actin was used as a loading control.

In summary, these new data provide the first in vivo evidence that aberrant HEF1 expression and changes in Aurora-A activation may be relevant to.genomic instability in cancer. Remaining work on Aim 1 will extend optimized protocols to additional samples, and will be completed early in 2009, as will analysis of HEF1, AurA, and centrosomal amplification/ aneuploidy in a mouse model of EOC.

KEY RESEARCH ACCOMPLISHMENTS:

First funding period

- We have optimized conditions for screening tumor lysates by Western blot for expression of all the proteins noted in the Aims.
- We have identified a statistically significant negative correlation between HEF1 overexpression and Aurora A overexpression.
- We have begun to analyze HEF1/AuroraA/centrosome expression in tissue microarrays.
- We have demonstrated a critical interaction between HEF1 and Aurora A that is necessary for Aurora A activation and mitotic progression.
- We have demonstrated that HEF1 interacts with Ajuba, with HEF1 and Ajuba synergizing to activate Aurora A.
- We have mapped the domains of HEF1 required for action at centrosome and focal adhesions, and we have shown that these are separable.

Second Funding period.

- We have fully mapped the domains for interactions between HEF1, AurA, and Ajuba.
- We have used 2D gel analysis to demonstrate that HEF1 interacts endogenously with AurA and Ajuba in a complex that predominates in G2 phase cells, but is also detected earlier in cell cycle.
- We have demonstrated mutual interdependence of HEF1 and AurA for protein stability, and for AurA activation.
- We have determined that AurA phosphorylation of HEF1 is important for HEF1 relocalization to focal adhesions at cytokinesis, and may contribute to the traction forces allowing resolution of cell separation.
- Current data do not support the idea that AurA contributes in a meaningful way to HEF1-dependent cell migration.
- We defined a completely novel role for HEF1-AurA interactions in regulation of cell cilia.

No cost extension.

- We have optimized conditions for measuring HEF1 and phospho-Aurora by immunohistochemistry
- We have applied this approach to establish a trend towards HEF1 overexpression and increased phospho-Aurora in serous EOCs
- We have found that HEF1 is commonly overexpressed in MOVCAR cell lines.
- We have determined that aberrantly reduced HEF1 expression (in a knockout model, crossed to transgene-induced oncogenes) is associated with an increased tendency to aneuploidy and centrosomal abnormalities.

REPORTABLE OUTCOMES:

The two funding periods of this project resulted in manuscripts published in Nature Cell Biology,

Cell Cycle[1, 15], and Cell[16]. We have described concepts and data emerging from these findings in 3 reviews[17-19]. Two additional manuscripts are in preparation for submission. In the past year, data from this project has been presented in invited talks at Louisiana State University Health Sciences Center, the ISN Forefronts Symposium on PKD in Montreal, Canada, the Gordon Research Converence on Basement Membranes, Biddeford, ME, Duke University Medical Center, Temple University Department of Biochemistry, Northwestern University, and a meeting at the NCI. The work will also be presented in 2 posters at the December 2008 annual meeting of the American Society for Cell Biology, and 1 poster at the 2009 American Association for Cancer Research. Data from this project has been used to support an RO1 grant application to the NIH for renewal of studies of HEF1 action in cancer (scored at 130, 1st percentile in summer 2008; awaiting Council review), and to support a second RO1 with Dr. Connolly as Principal Investigator, entirely focused on HEF1 actions in ovarian cancer (A1 submitted November 2008).

CONCLUSION:

Over the course of this study, we have greatly expanded mechanistic understanding of the function of HEF1, Aurora, and its partner Ajuba. We have illuminated the biology of ciliary resorption, a process linked to the control of centrosomal function and cell cycle progression. We have generated in vivo data indicating that aberrant expression of HEF1 in vivo induces centrosomal abnormalities and aneuploidy in one mouse cancer model, and are in the process of testing this idea in a second ovarian cancer model. We have optimized procedures for IHC evaluation of HEF1 and phospho-Aurora in ovarian tumors, and have suggestive data indicating a correlation between the two proteins in low and high grade serous tumors: further tumor assessment is in progress. In light of our results, and the growing number of papers emphasizing an important role for HEF1 in tumor cell invasion in multiple types of tumors [20-22], we believe that over the next year this protein will be established as an important biomarker in ovarian cancer progression.

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