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TITLE: Predicting Sensitivity of Breast Tumors to Src-Targeted Therapies through Assessment of Cas/Src/BCAR3 Activity

PRINCIPAL INVESTIGATOR: Amy H. Bouton, PhD

CONTRACTING ORGANIZATION: Rector and Visitors of the University of Virginia
Charlottesville, VA 22903-4833

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Amy H. Bouton, PhD

E-Mail: ahb8y@virginia.edu

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Rector and Visitors of the University of Virginia
1001 N Emmett St.
Charlottesville, VA 22903-4833

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14. ABSTRACT

Purpose: The purpose of this research is to assess the role of a signaling pathway comprised of the protein tyrosine kinase c-Src (Src) and two adaptor molecules, Cas and BCAR3, in promoting breast tumor growth, metastasis and therapeutic resistance toward Src-targeted small molecule inhibitors.
Scope: The proposed research employs 2- and 3-dimensional tissue culture models, transplantable mouse models of breast cancer, and analysis of human breast tumor samples.
Major Findings: Key results from the third year of support include (1) Further documentation using orthotopic tumor models demonstrating that BCAR3 is essential for tumor growth but that its interaction with Cas appears not to be required for this process; (2) the development of a robust clonigenic assay that we have used to show that BCAR3 controls cell proliferation/survival and dasatinib sensitivity; and (3) the acquisition of RNA-seq data that allow us to compare gene expression profiles as a function of BCAR3 expression under 3 culture conditions (plastic, 3D matrigel, and murine organoid cultures). The data we have generated are currently being prepared for publication; we expect to submit the manuscript by the end of the year.

15. SUBJECT TERMS
Breast tumor invasion, adhesion signaling, breast organoids, breast tumor initiation

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1. INTRODUCTION

The overall goal of this project remains to (1) understand the cooperating pathways through which signaling by the Cas/Src/BCAR3 signaling node drives tumor growth, metastasis and therapeutic resistance; and (2) be poised to begin clinical trials that will test BCAR3 expression as a predictor of response to the Src inhibitor dasatinib in combination with estrogen receptor (ER) and/or EGFR/HER2-targeted therapies. One exciting development associated with this project is that we have begun to collaborate with colleagues in the UVA School of Engineering and Applied Sciences to develop computational models examining the Cas/Src/BCAR3 signaling node in the context of a broader growth factor signaling landscape. Indeed, the complexity of the networks strongly suggests that optimal methods for pharmacological intervention may not be intuitive and may shift depending on expression of ER, EGFR, BCAR3, or other components of the network. This has not changed the overall scope or direction of the project, but instead provides a stronger platform upon which to place the preclinical data that we are generating when considering the clinical applications of this work.

2. KEY WORDS

Breast tumor invasions, adhesion signaling, breast organoids, breast tumor initiation.

3. ACCOMPLISHMENTS

We have made progress this year in several important areas: 1) further documentation using orthotopic tumor models demonstrating that BCAR3 is essential for tumor growth but that its interaction with Cas appears not to be required for this process; 2) the development of a robust clonigenic assay that we have used to show that BCAR3 controls cell proliferation/survival and dasatinib sensitivity; and 3) the acquisition of RNA-seq data that allow us to compare gene expression profiles as a function of BCAR3 expression under 3 culture conditions (plastic, 3D matrigel, and murine organoid cultures). The data we have generated are currently being prepared for publication; we expect to submit the manuscript by the end of the year.

We requested a no-cost extension for this project to cover the period from September 15, 2018 through September 14, 2019. As stated in my request, this was due predominantly to the length of time that it has taken to perform some of the mouse tumor studies because of the long latency period for tumor growth. The scope of the project has not changed; the remaining funds will be used to complete the proposed studies, submit the manuscript that is currently being prepared for publication, and respond to reviewer's comments.

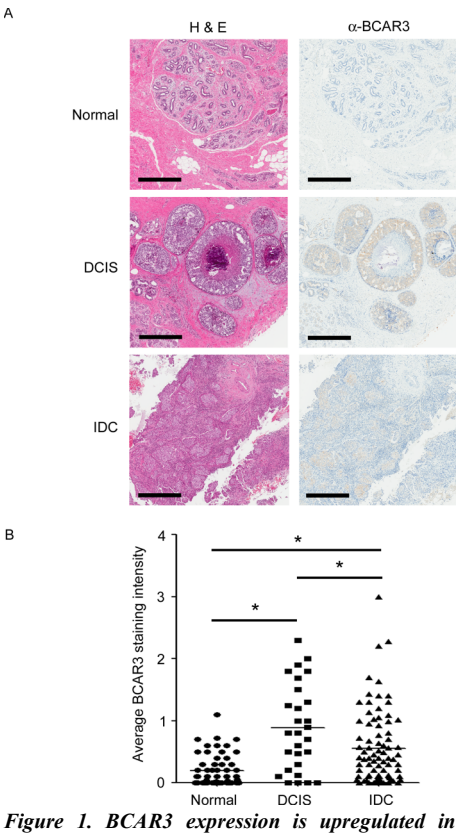

Figure 1. BCAR3 expression is upregulated in DCIS and Invasive Ductal Carcinoma (IDC). Normal, DCIS and IDC FFPE samples were stained for BCAR3 using a protocol validated by the Biorepository and Tissue Research Facility (BTRF) at UVA. (A.) Representative H&E and BCAR3-stained samples. Bar = 500 μm. (B.) BCAR3 expression was quantified by 2-3 investigators on 10 independent fields using a 4-point intensity scale for BCAR3 expression. *For 3-way comparison, $P<0.0001$ (one-way Anova); $P<0.0001$ (Normal to DCIS), $P=0.0188$ (DCIS to IDC) (2-tailed t test).

Specific Aim 1; Task 1. (see revised SOW, Yr 1 progress report)

Specific Aim 1; Task 2. *Expand the pilot study to evaluate BCAR3 expression in a larger cohort of clinical breast tumor samples.* This has been completed and data have been prepared as figures for a manuscript (Cross *et al.,* in preparation). They show that BCAR3 expression is elevated in both ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC)

(**Figure 1**), and that the majority of triple negative and ER+/PR+ breast tumors express some level of BCAR3 while the majority of normal mammary tissue does not (**Figure 2**).

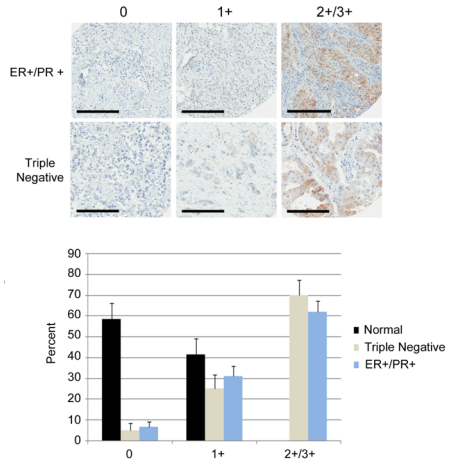

Figure 2. The majority of triple negative and ER+/PR+ invasive carcinomas express BCAR3 while the majority of normal mammary tissue does not). FFPE IDC samples obtained from the BTRF were stained for BCAR3 and quantified for BCAR3 expression as described in Figure 1. (Top) Representative images showing scoring for BCAR3 on a 0 to 3+ scale. Bar = 200 μm. (Bottom) The percentage of normal (black bars), triple negative (tan bars), and ER+/PR+ tissue samples containing regions of maximum BCAR3 staining intensity at the indicated levels. P<0.001, chi-squared test.

<u>**Specific Aim 2; Task 3**</u>. ***Determine whether dasatinib sensitivity can be modulated by BCAR3 expression and Cas/Src/BCAR3 signaling in tissue culture models.*** It has been quite a challenge to develop reproducible assays that allow us to unequivocally determine the effect of modulating BCAR3 expression on dasatinib sensitivity. However, we have had a recent breakthrough using a clonigenic assay in which cells are plated at low density, cultured for a period of 10 days, and stained with crystal violet. Under these conditions, there is a significant decrease in cell accumulation as a function of BCAR3 expression (**Figure 3**). MDA-MB-231 cells were subjected to this analysis in the presence of varying concentrations of dasatinib, ranging from 0.05uM to 0.1nM, and the IC50 was determined from the intensity of crystal violet staining (**Figure 4**). Using this approach, we found that the IC50 for dasatinib was decreased by 50 % (from 2nM to 1nM) in shBCAR3 compared to the control cells. This suggests that, as BCAR3 expression is reduced, the sensitivity to the Src inhibitor is increased. Since we have shown that BCAR3 knockdown decreases Src activity under those circumstances, this could mean that BCAR3 levels coordinately modulate Src activity and sensitivity to dasatinib. As mentioned above, we are working in collaboration with individuals who have an expertise in systems biology to develop computational models with which to predict these and other drug sensitivities.

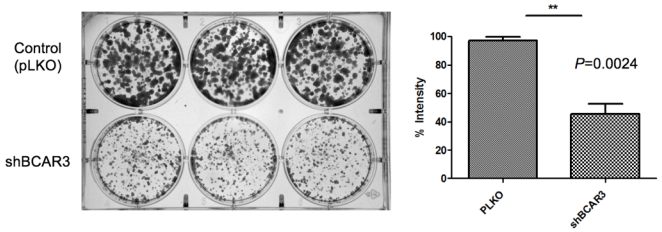

Figure 3. Accumulation of MDA-MB-231 cells over a 10-day period is significantly reduced under conditions of depleted BCAR3. MDA-MB-231 vector-control (pLKO) or shBCAR3 cells (see Fig. 5 for immunoblot) were plated at low density for 10 days with no media change, stained with crystal violet, and the signal quantified by Image J (NIH). *P*=0.0024, two-tailed t test.

<u>**Specific Aim 2; Task 4.**</u> ***Determine whether dasatinib sensitivity can be modulated by BCAR3 expression and Cas/Src/BCAR3 signaling in mouse models.*** As a first step in this aim, we sought to determine whether BCAR3 expression was required for primary tumor initiation or growth. This step is now completed and ready for publication. We first used an orthotopic transplantable model and found that shBCAR3 MDA-MB-231 cells were unable to initiate tumor growth (**Figure 5**). We subsequently used cells expressing a <u>conditional</u> knockdown allele for BCAR3 to investigate whether the growth of established tumors was similarly impacted under conditions of BCAR3 knockdown. Tumors were allowed to grow in the presence of BCAR3 until they reached 200

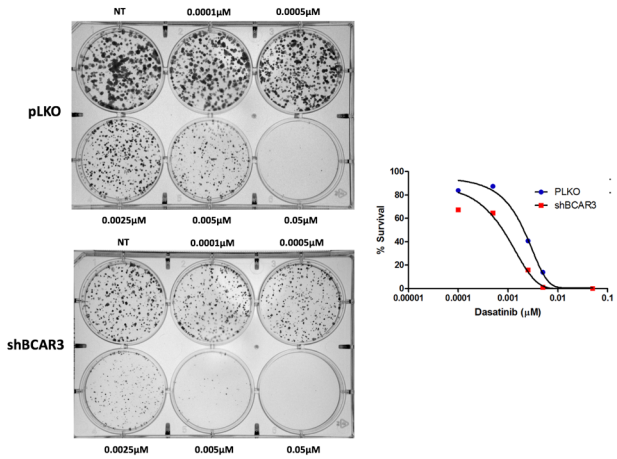

Figure 4. Knockdown of BCAR3 increases the sensitivity of MDA-MB-231 cells to dasatinib. MDA-MB-231 vector-control (pLKO) or shBCAR3 cells (see Fig. 5 for immunoblot) were plated at low density for 10 days with no media change in the presence of the indicated concentrations of dasatinib, stained with crystal violet, and the signal quantified by Image J (NIH). IC50 = 2 nM for pLKO control cells and 1 nM for shBCAR3 cells.

mm^3, after which the mice were fed doxycycline (Dox) in their drinking water to deplete BCAR3. The tumors continued to grow in animals injected with control cells (pLKO) and treated with Dox, while tumors established with the conditional shBCAR3 (shB3) cells failed to increase in size after Dox treatment was initiated (**Figure 6A**). Tumors isolated from animals injected with cells transduced with the pLKO vector stained positively for BCAR3, while cells transduced with pLKO-shBCAR3 had markedly reduced levels of BCAR3 (**Figure 6B**). Finally, in order to confirm that the defect in tumor growth was due to knockdown of BCAR3 and not an off-target effect of the small hairpin RNA, we performed a similar analysis to the first study using a cohort of cells in which BCAR3 is re-expressed in the shBCAR3 cells. For this experiment, we also expressed a variant of BCAR3 that was unable to bind to Cas to determine whether Cas binding was required for BCAR3-dependent tumorigenesis. As was the case for the experiments shown in Figure 5, shBCAR3 cells were unable to initiate tumor growth (**Figure 7**; see red line). Re-expression of wildtype BCAR3 rescued this defect (see blue line); in fact the tumors grew even faster than control cells, likely due to the overexpression of ectopic BCAR3 compared to the normal expression level of BCAR3 in MDA-MB-231 cells (data not shown). Surprisingly, the BCAR3 variant that is unable to bind to Cas was also able to promote tumor growth (green line), suggesting that BCAR3-Cas interactions are not required for tumor growth in this assay.

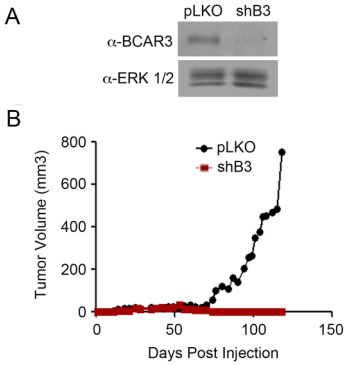

Figure 5. BCAR3 regulates initiation/progression of MDA-MB-231 xenografts. 10^6 vector-controlled (pLKO) or BCAR3-depleted (shB3) MDA-MB-231 cells (see panel A) were implanted into the mammary fat pads of nude mice. (B) Tumors were measured by caliper 2-3 times per week for the indicated times. Data are the median for 7 pLKO and 8 shBCAR3 tumors. P<0.05, F-test from log-linear repeated measures model.

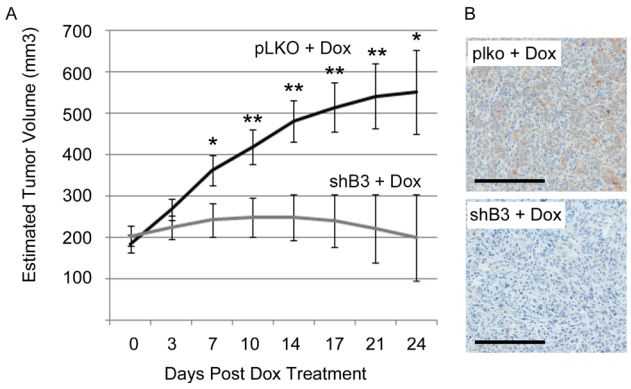

Figure 6. BCAR3 regulates growth of MDA-MB-231 tumors in nude mice. (A) 10^6 Dox-inducible vector-controlled (pLKO) or BCAR3-depleted (shB3) MDA-MB-231 cells were implanted into the 4th mammary fat pad of nude mice. Once tumors reached ~200mm^3, the animals were fed Dox for the duration of the experiment. Tumors were measured by caliper 2-3 times per week. Data presented are the mean +/- SEM for 10 pLKO and 8 shBCAR3 tumors. Using quadratic mixed effect models, **P*<0.03 and ***P*<0.01. (B) Representative images of FFPE tumor sections stained for BCAR3. Bar = 200μm.

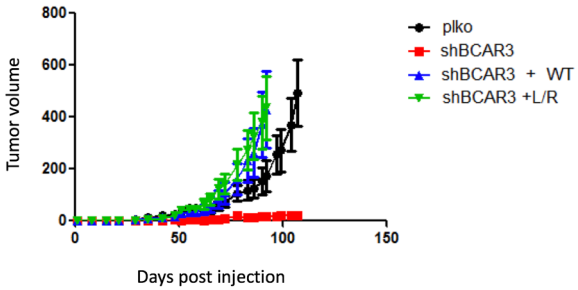

Figure 7. Ectopic BCAR3 rescues the defect in tumorigenicity exhibited by shBCAR3 MDA-MB-231 cells. 10^6 of the indicated MDA-MB-231 cell variants were implanted into the 4th mammary fat pads of nude mice. Tumors were measured by caliper 2-3 times per week for the indicated times. Data are the median for 10 pLKO (black), 9 shBCAR3 (red), 8 shBCAR3 + WT BCAR3 (blue) and 13 shBCAR3 + L744E/R748E (L/R; green) tumors. *P*=0.082 (one-way Anova).

<u>Specific Aim 3; Task 5.</u> ***<u>Test the hypothesis that Cas/Src/BCAR3 is downstream of HER family signaling in breast cancer cells using 2D and 3D systems.</u>*** We have taken a multi-pronged approach to this question. First, as was discussed in the year 2 progress report, we are using wildtype and BCAR3 knockout murine mammary organoids to identify proliferative signaling pathways that are impacted by the loss of BCAR3. We have now shown unequivocally that organoids established from BCAR3 knockout mammary tissue fail to bud or grow in size (**Figure 8**). Moreover, basal and luminal differentiation was normal in the BCAR3 knockout organoids as evidenced by cytokeratin 14 (biomarker for basal phenotype) and cytokeratin 8 (biomarker for luminal phenotype) staining (data not shown). Together these data provide support for the value of this model for studying BCAR3-dependent

proliferation/survival networks. This is being done through biochemical analysis of protein extracts, immunofluorescence microscopy, and by RNA-seq analysis. RNA-seq was performed on 3 independent samples of wildtype and BCAR3 knockout organoids using an Illumina NextSeq500, with an average of 44 million paired end reads per sample. We were able to subject these data to hallmark pathway analysis using Gene Set Enrichment Analysis (GSEA) and identified a number of pathways that are significantly (blue bars in **Figure 9**) up- or down-regulated in the knockout organoids as compared to wildtype organoids. These will be used to inform our next studies going forward using biochemical and immunofluorescent approaches.

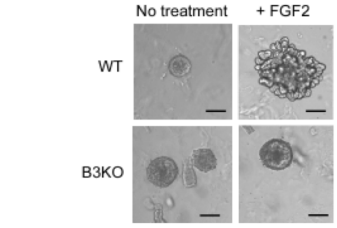

Figure 8. BCAR3 regulates organoid branching Cells were isolated from WT or global BCAR3 knockout (B3KO) mice [1], dispersed, and plated in matrigel culture in the presence or absence of 2 nM FGF2. Quantification showed a statistically significant decrease in branched structures generated from BCAR3 KO mammary epithelial cells. The size of the organoids was also significantly decreased in the BCAR3 KO as compared to WT organoids (quantification not shown).

In addition to the organoid system, we have been working to develop reagents to specifically study interactions between Cas/Src/BCAR3 and HER3 in light of a paper that was recently published describing HER3 as a direct binding partner of the BCAR3 SH2 domain (Li *et al.* 2017. *Nature Cell Biology* **19**:106-119). We are currently examining signaling downstream of neuregulin to identify pathways that are dependent on HER3-BCAR3 interactions using shBCAR3 knockdown cells and cells that re-express either wildtype or an SH2 binding mutation of BCAR3. Data from these experiments will inform future experiments that can be performed in the mouse.

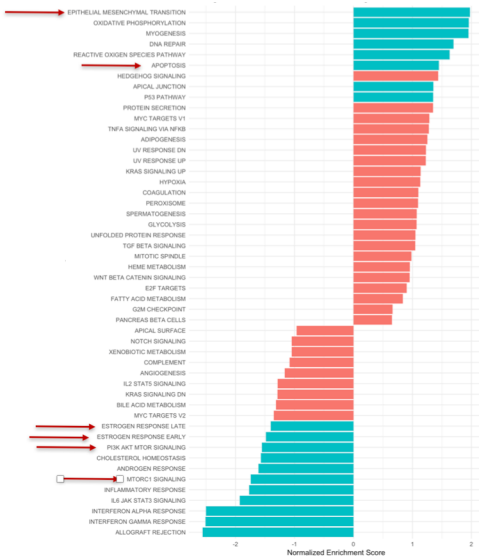

Figure 9. Hallmark pathways identified from Gene Sequence Enrichment Analysis (GSEA) are altered as a function of BCAR3 expression in mammary organoids. Transcriptome analysis was performed by RNA-seq on murine mammary organoids generated from wildtype and BCAR3 knockout mice. Red arrows depict pathways involved in proliferation/survival that are significantly up (bars to the right) or down (bars to the left) in BCAR3 knockout organoids. P <0.05 for the blue bars.

<u>**Specific Aim 3; Task 6**</u>. Our completed mouse tumor studies (see **Figures 5-7**) serve as a platform for the *in vivo* drug studies that are proposed in this task, and the data acquired from task 3 and 5 will be critical for designing the studies to provide maximum information regarding Src and EGFR family inhibitors. We will be working on these for the period covered by the no-cost extension.

4. IMPACT

The impact of our studies has not changed - while Src and EGFR family-targeted inhibitors have shown some activity in breast cancer patients, their lack of consistent efficacy has been somewhat surprising in light of the fact that elevated Src and EGFR family member kinase activities is a feature of many breast cancers. Thus, there is a pressing need for new approaches that can guide their application to patients who will most likely benefit from this class of drugs. With the extensive collection of clinical breast tumor samples that we have examined; the robust phenotypes that we have identified demonstrating a role for BCAR3 in tumor initiation/growth, invasion, and organoid branching; the genetic and cellular tools that we have developed; and the combined expertise of our team, we remain well-positioned to rapidly translate this work into the clinic and thus provide benefit to these patients.

5. CHANGES/PROBLEMS

Specific Aim 3. ***Investigate the role of Cas/Src/BCAR3 in EGFR/HER2 signaling and response to targeted therapies***. As mentioned in our prior progress report, additional evidence for a functional connection between BCAR3 and EGFR family members came to light through a publication by Li *et al.* (2017. *Nature Cell Biology* **19**:106-119). These authors reported that the SH2 domain of BCAR3 mediates direct binding to human epidermal growth factor receptor 3 (HER3), which recruits a second receptor-like tyrosine kinase (ROR1) and activates downstream YAP transcriptional programs. As a consequence of these new data, we have begun to collaborate with colleagues who have taken a systems approach to studying signals emanating from EGFR family proteins. In particular, Dr. Matthew Lazzara has extensive experience in ErbB/HER receptor biology, exploring its dysregulation in cancer and cancer cell sensitivity to targeted inhibitors of ErbB receptors (e.g. Monast, CS, Furcht CM, and Lazzara, MJ. 2012. *Biophysics J* **102**:2012-2021). Dr. Lazzara's group is now helping to develop computational models that incorporate the Cas/Src/BCAR3 signaling network into ErbB/HER signaling pathways. Predictions from these models will help to provide clearer insights into components of the pathways that lead to potential vulnerabilities that can be exploited therapeutically.

6. PRODUCTS

The following paper is being prepared for submission: Thomas, KS,* Cross, AM,* Arras, Janet,* Atkins, KA, Conaway, M and Bouton, AH. Breast Cancer Antiestrogen Resistance 3 (BCAR3) is differentially expressed in DCIS and invasive ductal carcinoma, and promotes breast tumor growth in murine models of breast cancer. ** denotes co-first authorship.*

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Participant	Role	Salary Support
Amy H. Bouton, Ph.D.	Principal Investigator	Unchanged
Kristen Atkins, M.D.	Co-Investigator	Unchanged
Mark Conaway, Ph.D.	Co-Investigator	Unchanged
Patrick Dillon, M.D.	Co-Investigator	Unchanged
Carol Gold, J.D., LLM	Consumer Advocate	Unchanged
Keena Thomas, M.S.	Laboratory Specialist	77.71%*
Janet Arras**	Graduate Student	100% effort, no salary (supported on T32 training grant)

*Ms. Thomas' support was reduced from 95% to 77.71% because she began performing experiments outside the scope of this grant as Janet Arras (Graduate Student) began taking on more responsibility. All other support remained unchanged.

** Ms. Arras joined the lab in March, 2017 and is devoting full-time to this project. She is currently supported by a T32 training grant from the NCI.

Other support: Nothing significant to report.

8. SPECIAL REPORTING REQUIREMENTS – None to report.

9. LIST OF APPENDICES - None