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14. ABSTRACT The nucleolar tumor suppressor ARF plays an important role in the tumor surveillance of human cancer. We have found that ARF expression is absent from highly proliferative prostate adenocarcinomas. This correlates with the normal expression of the p53 tumor suppressor gene indicating that ARF loss could be a contributing factor for prostate cancer initiation and/or progression. We have found that ARF-mull mice develop prostatic lesions by 9 months of age (2/10), but die of sarcoma or lymphoma. We have generated and are monitoring prostate specific ARF and ARF/p53 knockout animals for the development of prostate lesions avoiding the complication of genomic loss of these tumor suppressors. While our mouse prostate epithelial cultures have not grown well, we have taken two additional approaches to assess ARF's role in prostate growth control. First, we have lentiviral shRNA constructs which can knockdown basal ARF levels for use in commercially available normal human prostate epithelial cell lines. Second, we have developed a protocol to isolate polysomes from freshly isolated whole mouse prostates. Both of these techniques will allow us to monitor polysome mRNA association in the absence of ARF. While we have encountered difficulties in this first year, we have developed new techniques to allow the research project to progress toward the approved goal of biomarker development.					
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INTRODUCTION:

In numerous human cancers, the frequency of loss of the ARF tumor suppressor is second only to mutation of p53^{1,2}, providing critical evidence of ARF's role in preventing tumorigenesis throughout the body, irrespective of cell type. However, the result of ARF loss in the prostate is currently understudied. Our preliminary data using human samples from Washington University indicates that prostate adenocarcinomas typically maintain wild type p53 levels (97%), but lose ARF (96%) expression. These data suggest ARF loss may play a role in the development and/or progression of prostate cancer. Mechanistically, we have previously shown ARF to be a critical regulator of ribosome biogenesis³⁻⁵.

The ultimate goal of this proposal is to identify new downstream therapeutic targets required for the development of prostate cancer through the identification of polysome-associated mRNAs in *Arf*^{-/-} prostate epithelial cells. *We hypothesize that the loss of Arf in prostate epithelial cells will lead to an increase in ribosome production and an alteration in the pool of polysome-associated mRNAs towards transcripts that encode proteins critical to prostate tumorigenesis. As such, these translated mRNAs will produce proteins that are required for prostate cancer development and therefore, these proteins will be potential novel targets for prostate cancer treatment or diagnosis.*

BODY:

As outlined in the approved Statement of Work, we focused our energies on the tasks planned for Months 1-12. These included experiments outlined in Tasks 1, 2, and 3. In this first annual progress report, we detail the progress and results from these studies.

Task 1. To determine the effects of *Arf* loss on Prostate Intraepithelial Neoplasia formation (Months 1-24):

- a. Examining the effects of prostate-specific *Arf* loss on prostate cancer formation and progression.

During the first year of the grant we sought to determine the effects of *Arf* loss on PIN formation in genomic *Arf*^{-/-} mice. Our preliminary studies performed in conjunction with the Washington University School of Medicine Small Animal Pathology Core were proven wrong due to tangential cuts in the prostate tissue sections examined. At the time of the grant submission we had promising preliminary results of 3 of 3 *Arf*^{-/-} and 0 of 4 wild type mice developing Prostate Intraepithelial Neoplasia (PIN). However, further submission of mice the Small Animal Pathology Core found PIN lesions 6 of 7 *Arf*^{-/-} and 5 of 5 wild type mice. PIN lesions in C57BL6 mice (background for *Arf*^{-/-} mice) have never been reported in the literature. We asked Dr. Jeff Arbeit in the department of Surgery here at Washington University for his expertise in mouse prostate tumor development. With his help, we have regenerated a cohort of 10 *Arf*^{-/-} and 12 wild type mice with the proper tissue section preparation as described⁶. Using the prostate "tree" layout, we were able to determine that 0 *Arf*^{-/-} and 0 wild type developed PIN (**Figure 1**). However, 2 of the *Arf*^{-/-} mice developed prostatic lesions at 9 months of age (**Figure**

2). One grew in the ventral lobe of the mouse and was characterized by a high proliferative marker, Ki-67. While the cells lacked basal (K5), luminal (K8), and neuroendocrine (synap) markers, the cells were Androgen Receptor (AR) positive (**Figure 2**, left column). The second lesion occurred in the anterior lobe of a different mouse. This lesion appears to be a fibrosarcoma by hematoxylin and Eosin (H&E) staining of unknown origin as it does not stain for any of the prostate markers (**Figure 2**, right column).

As discussed in the original proposal, we anticipated a problem using male mice with a genomic loss of *Arf* who die by 9 months of age due to sarcoma or lymphoma development. We have produced a mouse colony containing males with a prostate specific deletion of *Arf* by crossing *Arf^{fl/f}* mice with ARR2Pb-Cre mice. These Pb-Cre/*Arf^{fl/f}* and *Arf^{fl/f}* mice will be sacrificed beginning at 15 months up to 24 months of age to assess prostatic lesion development and avoid the complications of sarcoma and lymphoma development. We currently have four (4) Pb-Cre/*Arf^{fl/f}* and ten (10) *Arf^{fl/f}* male mice and continue to produce litters to expand the number of male mice to examine for PIN formation.

In addition, we have produced a colony of Pb-Cre/*Arf^{fl/f}* /*p53^{fl/f}*, Pb-Cre/*p53^{fl/f}* and *Arf^{fl/f}*/*p53^{fl/f}* mice to examine for PIN and prostate cancer development. We currently have two (2) Pb-Cre/*Arf^{fl/f}* /*p53^{fl/f}*, twenty (20) *Arf^{fl/f}* /*p53^{fl/f}* male mice and continue to produce litters to expand the number of male mice to assess for prostate cancer development as described above.

Task 2. To test the effects *Arf* loss on prostate epithelial cell growth (Months 1-9):

- a. What are the effects of *Arf* loss on ribosome biogenesis (Months 1-4)?
- b. What are the effects of *Arf* loss on protein synthesis and cell size (Months 5-6)?
- c. What is the dependence of the cellular growth on ARF expression (Months 6-9)?

During the first year of the grant, we have encountered problems with the murine prostate epithelial cell line we developed. While epithelial cells are present in the initial explants from freshly isolated mouse prostates (**Figure 3**, arrows) as demonstrated by positive staining for cytokeratin 8, e-cadherin (**Figure 4**), the population is overtaken by a stromal smooth muscle actin (SMA) positive cell population. The initial subpopulation of epithelial cells is luminal in origin as indicated by the Pan cytokeratin, cytokeratin 8, and E-cadherin positive staining and lack of basal and neuroendocrine markers cytokeratin 5 and synaptophysin; respectively (**Figure 4**). As stated in the original proposal, these cells depend on androgens for growth as bicalutamide and flutamide, both inhibitors of Androgen Receptor (AR) signaling; inhibit their growth (data not shown). We have tried numerous culturing techniques including plating the cells on collagen and PRIMARIA (Fisher Scientific) specialty tissue culture plastic. We have used Stem Cell Technologies' Mouse Prostate Epithelial Cell Isolation Kit to no avail. We have also combined techniques in various combinations to no avail. For example, using the Mouse Prostate Epithelial Cell Isolation kit and plating the cells in Matrigel does not allow the epithelial cells to grow out from the stromal cells we continually see dominate the cultures. In addition, we have used several media formulations for epithelial and prostate cell culture. These have not helped to promote the specific growth of prostate epithelial cells in our cultures.

We have not abandoned attempts to produce a pure population of mouse prostate epithelial cells and will continue to work on the proper conditions to remove the stromal cell population and allow the epithelial cells to grow. Our current efforts focus on using an Alexafluor-488 labeled E-cadherin antibody to sort the population of freshly isolated mouse prostate epithelial cells and using various plating techniques and to help their growth.

However, we wish to perform the experiments listed in the statement of work. To this end we have turned to normal human prostate epithelial cells which are available from several commercial sources including Lonza (Basel, Switzerland) and Lifeline Technologies (Walkersville, MD). As shown in **Figure 5A**, p14ARF is expressed at very low levels in human prostate epithelial cells. This is not unprecedented as we have previously shown that wild-type mouse embryo fibroblasts (MEFs) have very low basal ARF that does have a role in regulating nucleolar function in the cell⁵. Therefore, the low level of ARF in normal human prostate epithelial cells (hPrEP) may be acting in a similar fashion. Using lentiviral siRNA constructs targeting p14ARF already obtained from the RNAi Core at Washington University, we will knockdown ARF levels in these cells. We have tested the lentiviral siRNA constructs in a p14ARF positive cell line. As shown in **Figure 5B**, lentiviral constructs A9, A11, and A12 reduced the steady state mRNA levels of p14ARF. We are currently validating the knockdown at the protein level. These constructs will be used to perform all the experiments listed in the approved statement of work. This does not alter the approved statement of work, we are simply substituting a human prostate epithelial cell line for the mouse cell line.

Task 3. To determine the effect of loss of the *Arf* tumor suppressor on the pool of polysome associated mRNA in prostate epithelial cells. (Months 6-24)

- a. What are the effects of *Arf* loss on Polysome-Associated mRNAs in prostate epithelial cells (Months 6-9)?
- b. Validation of the mRNA's Identified in the Polysome Microarray (Months 10-16).
- c. Dependence of the Identified mRNA's on ARF expression (Months 14-18).
- d. Analysis of Validated mRNAs in prostate cancer tissues (18-24).

The work on this task has also been delayed due to the problems we are having with the mouse prostate epithelial cell line generation. We plan on taking two approaches to perform the experiments detailed in the statement of work. The first is to use the lentiviral siRNA mediated p14ARF knockdown in normal human prostate epithelial cells as detailed in *Task 2* above. Using normal human prostate epithelial cells lacking p14ARF, we will perform the polysome associated RNA Microarray. We will validate the changes in human normal prostate epithelial cells lacking ARF at the mRNA and protein level. This approach will increase our chances of finding candidate biomarkers relevant to human disease and does not alter the approved statement of work.

The second approach is to perform the polysome associated mRNA Microarray analysis on freshly isolated mouse prostates. Briefly, the mice will be cardiac perfused with PBS containing cycloheximide followed by *RNAlater* (Applied Biosystems) containing cycloheximide to stabilize polysomes (ribosome-mRNA complexes) similar to what has been previously described⁶. One half (½) of the tissue corresponding to one of each of the 4 lobes (A, V, L, and D) will be isolated, tamped to remove secretions, ground in a tissue grinder, and subjected to cytosolic polysome fractionation. The peaks corresponding to the polysome will be collected and RNA isolated with RNA-solv (Omega-BioTek). Total cellular RNA will be isolated from the other ½ of the prostate (1 lobe each A, V, L, and D). Quadruplicate samples for the polysome RNA and duplicate samples for total RNA will be taken for Illumina Mouse 8 array analysis. As shown in **Figure 6A**, we are able to isolate polysomes from freshly isolated mouse prostates. Interestingly while *Arf*^{-/-} prostates have lower 40S, 60S, and 80S ribosome peaks compared to *Arf*^{+/+}, the polysomes in *Arf*^{-/-} are significantly higher. Importantly, the

difference between the translational profiles from *in vitro* cell culture and *in vivo* tissue is drastic (compare **Figures 6A** and **6B**). Using this new technique will provide a more accurate picture of which mRNAs are translated *in vivo* in the prostate as the tissue moved towards carcinogenesis.

KEY RESEARCH ACCOMPLISHMENTS:

- We have determined that *Arf*^{-/-} mice develop prostatic lesions (2 of 10 *Arf*^{-/-} vs 0 of 10 *Arf*^{+/+}) at 9 months of age, but die from lymphoma or sarcoma at that time.
- We have developed four new mouse colonies for prostate cancer modeling – Pb-Cre/*Arf*^{f/f}, Pb-Cre/*p53*^{f/f}, Pb-Cre/*Arf*^{f/f}/*p53*^{f/f}, and *Arf*^{f/f}/*p53*^{f/f} which will allow the mice to be followed beyond the lifespan of the corresponding genomic knockouts.
- We have developed a wild-type and *Arf*^{-/-} SMA-positive prostatic stromal cell line to use as a tool to assess stromal contributions to prostate cancer development.
- We have identified 3 siRNAs to use to knockdown p14ARF in human prostate epithelial cells.
- We have developed a new protocol for examining polysome levels and polysome associated mRNAs from freshly isolated mouse prostates.

REPORTABLE OUTCOMES:

Manuscripts/Abstracts: None

Presentations:

“Protein Translation and Prostate Cancer”	Prostate Cancer Research Group Washington University School of Medicine Saint Louis, MO
“Arf Loss Alters the Translatome to Permit Transformation”	Breast Cancer Research Group Washington University School of Medicine Saint Louis, MO
“Polysome Associated mRNA – Priming Cells for Transformation”	Microarray Workgroup Washington University School of Medicine Saint Louis, MO
“The mTOR Pathway: Translational Control and Cancer.”	Cancer Biology Pathway Washington University School of Medicine Saint Louis, MO

Patents/Licenses: None

Animal Models:

Pb-Cre/*Arf*^{f/f} – Specifically deletes the *Arf* tumor suppressor in the prostate allowing the mice to be followed for the development of prostate lesions beyond the 9 month lifespan of genomic *Arf*^{-/-} mice.

Pb-Cre/ $p53^{f/f}$ – Specifically deletes the p53 tumor suppressor in the prostate allowing mice to be followed for the development of prostate lesions beyond the 5 month lifespan of genomic $p53^{-/-}$ mice. These mice have been reported in the literature, but this is a newly developed colony at Washington University.

Pb-Cre/ $Arf^{f/f}/p53^{f/f}$ – Specifically deletes the Arf and $p53$ tumor suppressors in the prostate allowing the mice to be followed for prostate lesion development beyond the lifespan of genomic knockouts of either gene alone.

$Arf^{f/f}/p53^{f/f}$ – Wild type control mice for experiments using Pb-Cre/ $Arf^{f/f}/p53^{f/f}$ mice.

Applied for Funding:

2010 Department of Defense Peer Reviewed Cancer Research Program Idea Award - “Neurofibromin-regulated Protein Translation in NF1” (CA100049)

2010 Department of Defense Prostate Cancer Research Program Exploration: Hypothesis Development Award – “Translational Alterations in c-Myc Driven High Grade PIN Lesions In Vivo” (PC100153)

2010 Siteman Cancer Center Washington University Developmental Research Award in Prostate Cancer – “Translational Alterations in c-Myc Driven High Grade PIN Lesions In Vivo”

Applied for Employment:

Promotion to Research Assistant Professor at Washington University School of Medicine

Received Research Opportunities:

2010 Siteman Cancer Center, Research Associate Member

CONCLUSIONS:

During the first year of this project we have made significant progress on the Tasks outlined for months 1-12. While it has been difficult, but we have found alternative approaches for each of the problems encountered and have begun working on each task. These alternative approaches do not alter the approved statement of work. They only alter the starting material for the experiments using human prostate epithelial cells instead of mouse prostate epithelial cells or using freshly isolated mouse prostates rather than a cell line. The tasks outlined can be completed using these as substitutes while we work to obtain a pure mouse prostate epithelial cell culture.

First, we have shown that 20% of $Arf^{-/-}$ mice develop prostatic lesions. However, these mice die at 9 months of age due to disease unrelated to the prostatic lesions. To circumvent this problem, we have developed four (4) new mouse models for prostate tumor formation, Pb-Cre/ $Arf^{f/f}$, Pb-Cre/ $p53^{f/f}$, $Arf^{f/f}/p53^{f/f}$, and Pb-Cre/ $Arf^{f/f}/p53^{f/f}$. These prostate specific animal models will allow us to assess ARF's specific role in prostate tumorigenesis.

Second, we have found 3 lentiviral constructs that can be used to knockdown ARF mRNA expression in human prostate epithelial cells. This provides us with a tool to assess for the first time ARF's role in ribosome biogenesis in human cells. Additionally it will allow us to perform polysome associated RNA microarrays to assess ARF's effect on the proteins translated in human prostate cells.

Third, we have developed a new technique to assess the role of ARF in polysome formation *in vivo*. Using freshly isolated mouse prostates, we can assess *in vivo* the polysome associated mRNAs upon ARF loss. Importantly, our data shows that *in vivo* polysomes are drastically different than those reported for cell culture. The homeostatic translational profile is much lower in tissues relative to continually proliferating cell culture and will give a more accurate view of what mRNAs are translated in the prostate.

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APPENDICES: None

SUPPORTIN DATA: See below (Figures 1-6)

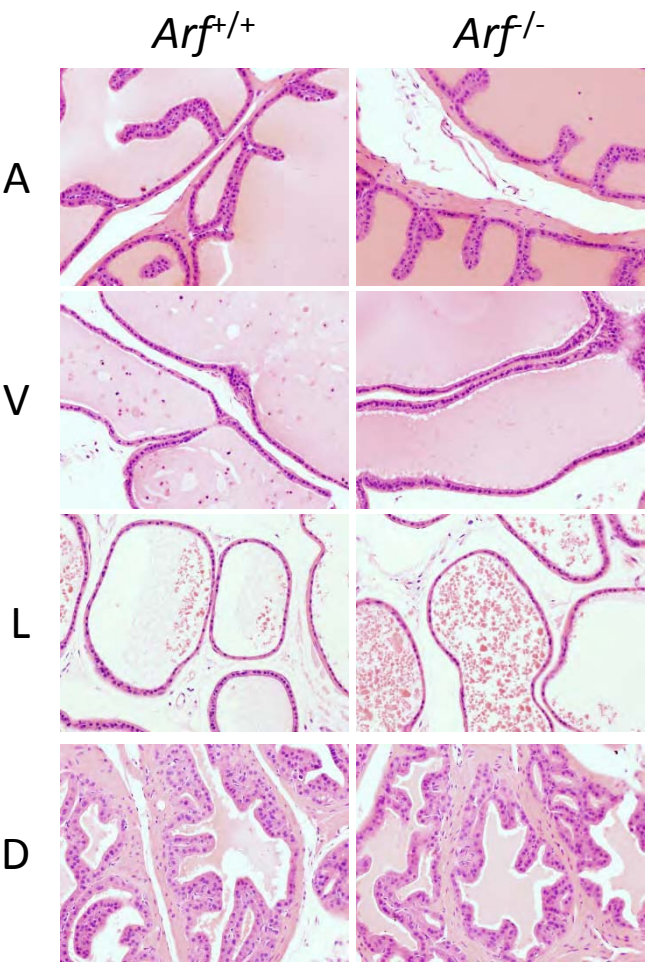


Figure 1. Representative H&E Stain of *Arf*^{+/+} and *Arf*^{-/-} Mouse prostates. Thirty-nine (39) week old mice were sacrificed and their prostates removed, fixed, sectioned, and stained with Hematoxylin and Eosin. 20x magnification; A, Anterior; V, Ventral; L, Lateral; D, Dorsal.

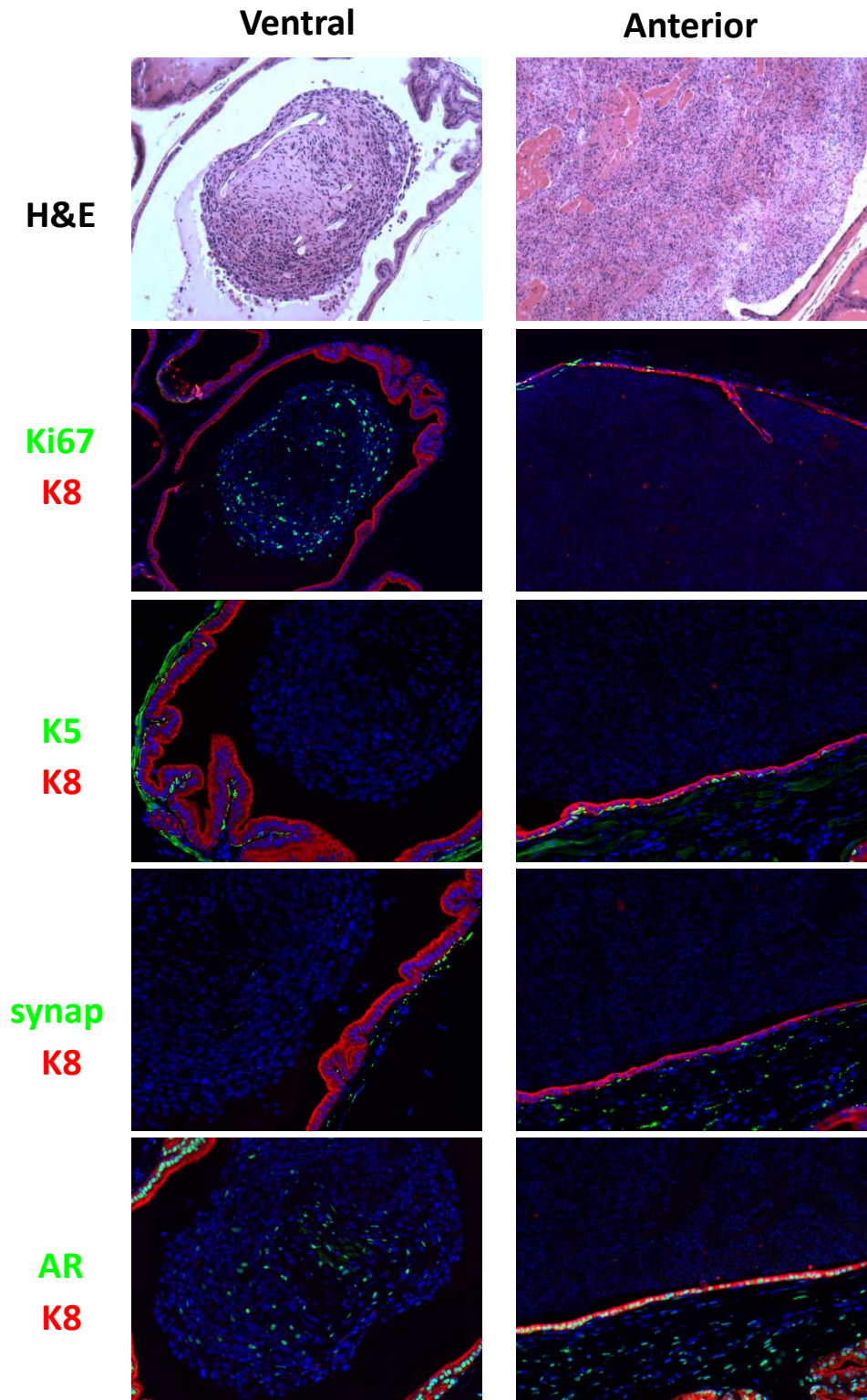


Figure 2. Characterization of *Arf*^{-/-} Prostate Lesions. Thirty-nine (39) week old mice were sacrificed and their prostates removed, fixed, sectioned, and stained as indicated. One mouse had a ventral lesion and the second had an anterior lesion. Nuclei are stained with DAPI (blue) except Hematoxylin & Eosin (H&E) panels. K8, cytokeratin 8; K5, cytokeratin 5; synap, Synaptophysin; AR, Androgen Receptor.

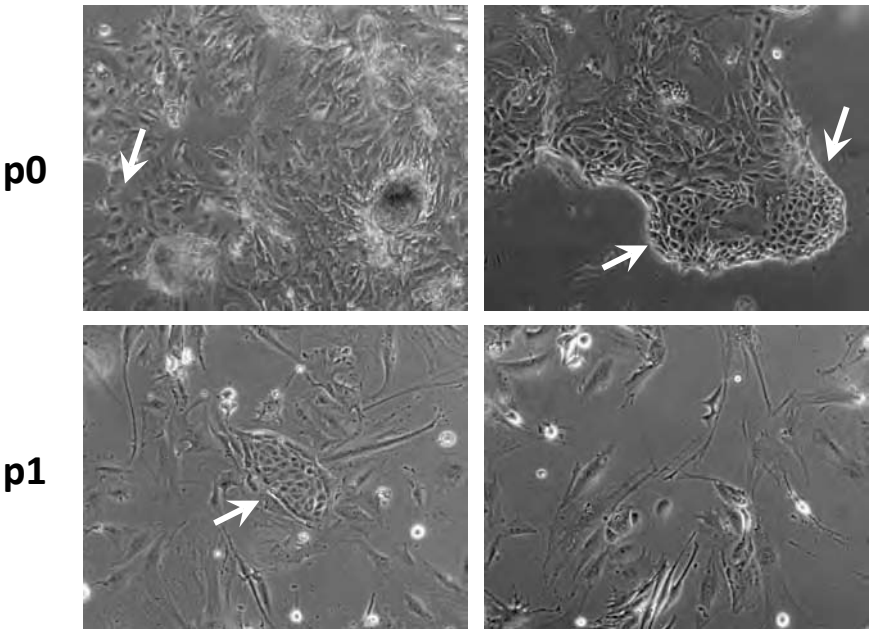


Figure 3. Characterization of *Arf*^{-/-} Prostate Cell Cultures. Phase contrast microscopy of freshly explanted cultures (p0) show a mixed population of cells with epithelial cells present (arrow). A single passage (p1) results in a loss of epithelial cell morphology in the cultures.

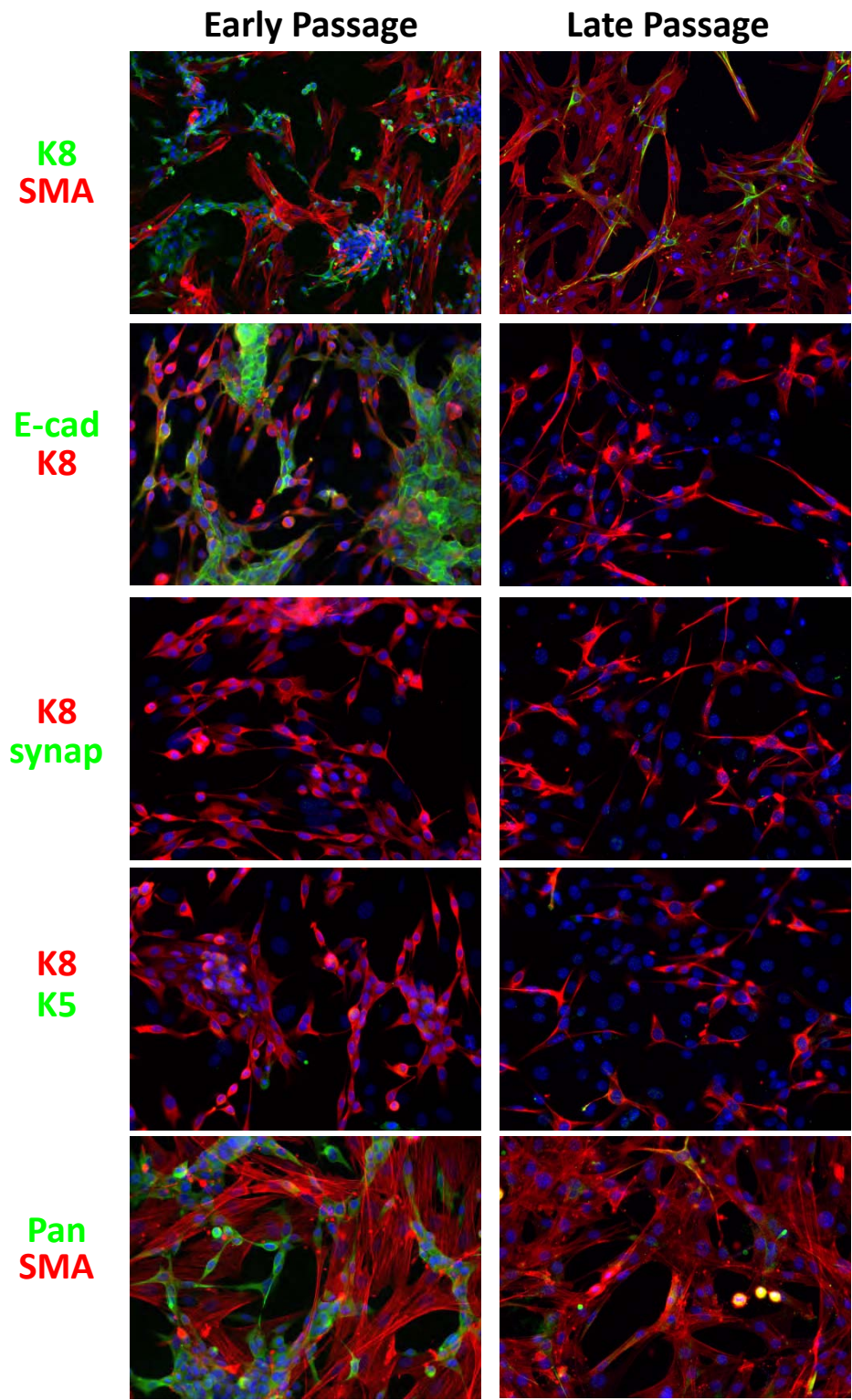


Figure 4. Characterization of *Arf*^{-/-} Prostate Cell Cultures. Early passage (<6) and late passage (>12) *Arf*^{-/-} mouse prostate cells were fixed in formalin and stained for the indicated prostate epithelial markers (K8, cytokeratin 8; SMA, smooth muscle actin; E-cad, E-Caherin; K5, cytokeratin 5; synap, Synaptophysin; Pan, Pan cytokerain. All panels have nuclei stained with DAPI. 10X magnification.

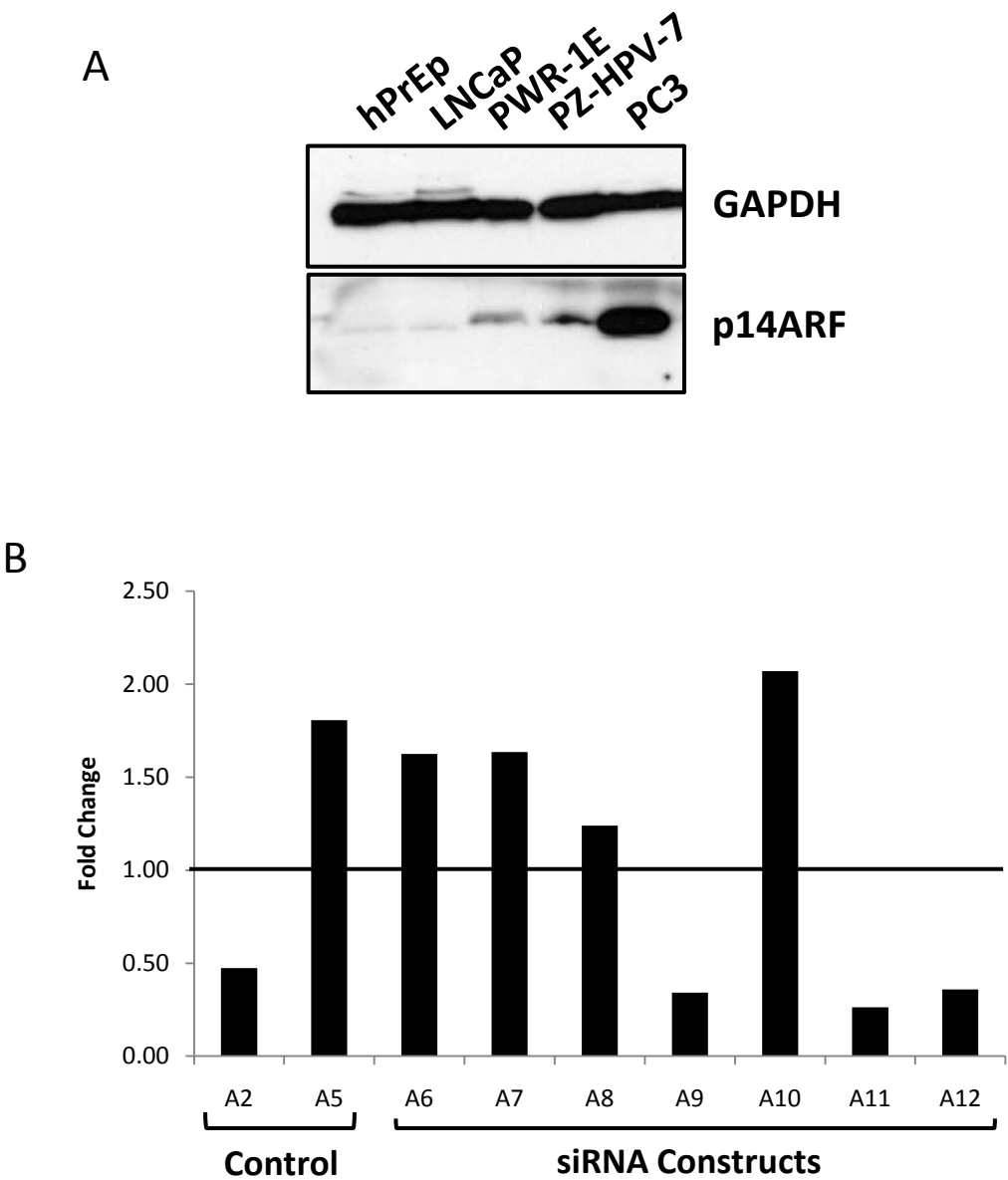


Figure 5. p14ARF levels in human prostate cell lines. (A) Western blot analysis of p14ARF protein levels in human prostate epithelial cell lines. hPrEp, normal human prostate epithelial cells. **(B)** Real Time PCR analysis of p14ARF mRNA levels normalized to HPRT and relative to uninfected cells upon lentiviral delivered siRNA knockdown. Fold Change is calculated by the $2^{-\Delta\Delta Ct}$ method.

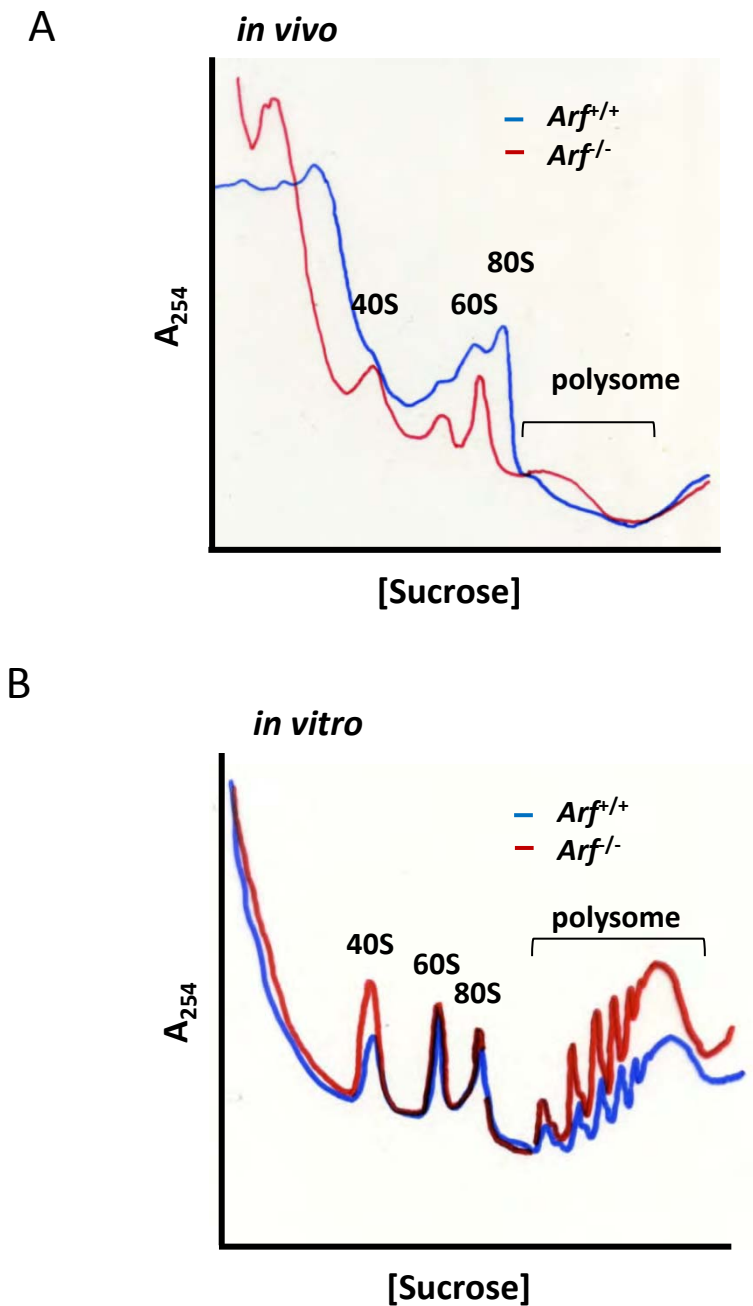


Figure 6. Effects of ARF loss on polysome formation *in vivo*. (A) Cytosolic ribosome formation was measured on freshly isolated wild type and $Arf^{-/-}$ mouse prostates. One-half of each prostate consisting of 1 lobe each, Anterior, ventral, lateral and dorsal was subjected to polysome analysis. (B) Cytosolic ribosome formation was measured on equal numbers (3×10^6) of wild type and $Arf^{-/-}$ mouse embryo fibroblasts.