Chapter

Antibody Therapy Targeting Cancer-Specific Cell Surface Antigen AGR2

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Abstract

For anterior gradient 2 (AGR2), normal cells express the intracellular form iAGR2 localized to the endoplasmic reticulum while cancer cells express the extracellular form eAGR2 localized on the cell surface and secreted. Antibodies targeting $eAGR2^+$ cancer cells for eradication will spare normal cells. Two AGR2 monoclonal antibodies, P1G4 and P3A5, were shown to recognize specifically $eAGR2^+$ pancreatic tumors implanted in mice. In addition, P1G4 showed enhancement in drug inhibition of tumor growth. Human:mouse chimeric antibodies of IgG1, IgG2, IgG4 were generated for both antibodies. These human IgG were shown to lyse $eAGR2^+$ prostate cancer cells in vitro with human serum. AGR2 has an important function in distal spread of cancer cells, and is highly expressed in prostate, pancreatic, bladder metastases. Therefore, immunotherapy based on AGR2 antibody-mediated ADCC and CDC is highly promising. Cancer specificity of eAGR2 predicts possibly minimal collateral damage to healthy tissues and organs. Moreover, AGR2 therapy, once fully developed and approved, can be used to treat other solid tumors since AGR2 is an adenocarcinoma antigen found in many common malignancies.

Keywords: anterior gradient 2, adenocarcinoma antigen, solid tumors, metastasis, chimeric AGR2 antibodies, antibody enhancement of drug inhibition

1. Introduction

The adenocarcinoma antigen anterior gradient 2 (AGR2) is expressed by prostate, pancreatic and bladder cancer as well as many other solid tumor types. In 2018, close to 1.3 million new cases of prostate cancer worldwide were diagnosed and 360,000 deaths were recorded, mostly in the developed countries [1]. In the same year, nearly 460,000 new cases of pancreatic cancer were diagnosed [2]. Patients with pancreatic cancer seldom exhibit symptoms until at advanced stages making 5-year survival dismal. Bladder cancer represents only 3% of global cancer diagnoses, and 5-year survival decreases from >75% to 5% when the cancer has metastasized [3].

Since the FDA approval of IFNα2 in 1986, a number of immunotherapeutic agents have been developed for cancer treatment. In prostate cancer, antigens such as PSA, PAP, PSCA, MUC1, PAGE/GAGE were used to impart T cell-mediated immunity [4]. Lack of consistent success could be partly attributed to non-prostate

specific expression of these proteins. PSCA (prostate stem cell antigen), for example, is misidentified as a stem cell marker, and is expressed by the bladder, colon, kidney and stomach as well [5]. Vaccines against PSA (prostate-specific antigen) in PROSTVAC-VF and PAP (prostatic acid phosphatase) in Provenge were used to prime immune cells. However, inconsistent trial results with modest survival benefits were reported [6]. Strategies to target immune checkpoints (CTLA-4, PD-1, PD-L1) with the intention to amplify T cell responses in eradicating tumors have not been particularly successful in prostate cancer, with one of the main side effects being immune-related adverse events with tissue damage caused by overly activated T cells [6]. These potential therapies are beset by response monitoring although a subset of patients with advanced disease did show some benefits. There are even fewer similar types of immunotherapeutic approaches developed for pancreatic and bladder cancer, which is largely due to fewer suitable targets identified. Immunotherapy based on antibodies, on the other hand, would not require tinkering the immune system to achieve an outcome. Tumor-associated antigens (TAA) [7] constitute a pool of candidates for targeted cancer therapies. Antibodies raised against TAA mediate cancer cell killing through antibody-dependent cellular cytotoxicity (ADCC) by recruiting cytotoxic T cells [8] and complement-dependent cytotoxicity (CDC) by assembling complement components into a membrane attack complex [9]. The antibody-bound cancer cells are lysed by T-cell secreted enzymes and water uptake through a perforated cell membrane, respectively. A major complication is the cancer non-specificity of most TAA identified to date, because other normal cell types also express these TAA leading to unintended collateral damage of healthy tissue. Therefore, the quest for a truly cancer-specific targetable molecule is an ongoing endeavor.

Our work in Urologic Cancer Biomarker Development identified AGR2 as a TAA for prostate cancer. AGR2 is present in prostate cancer cells but absent in the normal luminal cells [10, 11]. Similarly, AGR2 expression is detected in various cancers including pancreatic [12], breast [13], lung [14], colorectal [15], oral [16], subsets of ovarian [17], and bladder [18]. What makes AGR2 attractive for cancer therapy besides its ubiquity in solid tumors is its differential subcellular localization between cancer and normal cells [19] as we demonstrated previously for bladder cancer [18]. Intracellular iAGR2 is localized to the endoplasmic reticulum (ER) of normal cells where, as a protein disulfide isomerase, it functions in protein folding [19]. Extracellular eAGR2 is localized to the cell surface of and secreted by cancer cells. Thus, eAGR2 is a unique TAA that it is not found on normal cells.

Antibodies targeting eAGR2 on cancer cells would thus spare normal cells as the iAGR2 antigen is cell interior. We have generated mouse monoclonal antibodies, P1G4 (mIgG1) and P3A5 (mIgG2a), recognizing two epitopes of AGR2 [20]. The use of mouse monoclonal antibodies for therapeutics is problematic. Mouse antibodies, besides being immunogenic in human, do not interact efficiently with human immune system components. To overcome these drawbacks, we have replaced the constant domains of AGR2 antibodies by the analogous human constant domains via recombinant DNA technology [21], generating human:mouse chimeric hIgG1, hIgG2, hIgG4 for both P1G4 and P3A5 for potential therapeutic development [22].

With these chimeric human:mouse antibodies, direct antigenic stimulation of T cells via CAR-T cell therapy can potentially also be achieved [23]. We could link via the engineered restriction enzyme sites the antigen-binding V_H and V_L domains of the AGR2 antibodies to T cell activator molecules for triggering response upon binding of the T cells via eAGR2 on the cancer cell surface. In the future, one might be able to use patient-derived induced pluripotent stem (iPS) cells to differentiate into dendritic cells in vitro (by bone marrow factors and interaction with bone marrow

stromal cells) rather than leukapheresis to isolate dendritic cells from patients. The derived dendritic cells can then be stimulated with AGR2 antigens. The use of AGR2 to transduce dendritic cells via expression vectors to generate cytotoxic T lymphocytes capable of lysing AGR2$^+$ (colorectal) cancer cells has been reported [24].

In this review, we will discuss the role of AGR2 in various cancers, the development and therapeutic evidence of chimeric AGR2 antibodies, and the future use of AGR2 in biomarker and therapeutic applications.

2. AGR2 as a cancer biomarker

We used comparative transcriptomic analysis between sorted CD26$^+$ prostate cancer cells from a Gleason pattern 3 (well-differentiated glandular adenocarcinoma) tumor focus and CD26$^+$ luminal cells of benign glands to identify differentially expressed genes that encode secreted proteins for use as urine biomarkers [10]. AGR2 was the top candidate with elevated expression in cancer cells, which was verified by immunohistochemistry (**Figure 1**). A similar analysis was carried out between CD9$^+$ urothelial cancer cells and CD9$^+$ normal urothelial cells [25]. In this case, AGR2 was down-regulated in cancer. AGR2 is expressed by urothelial cells (iAGR2 for normal cells) and was found absent in 75% of bladder cancer cases [18]. Although the AGR2 gene contains a leader signal peptide characteristic of secreted proteins, in normal cells iAGR2 is retained in the ER (via a *C*-terminus ER-retention motif). Over-expression of AGR2 in cancer cells provides a possible explanation for its secretion due to saturation of ER anchorage [26]. For example, urothelial cells were immunostained for AGR2 at moderate intensity *vs*. prostate cancer cells. The immunostaining corroborated a 35-fold difference in expression levels measured

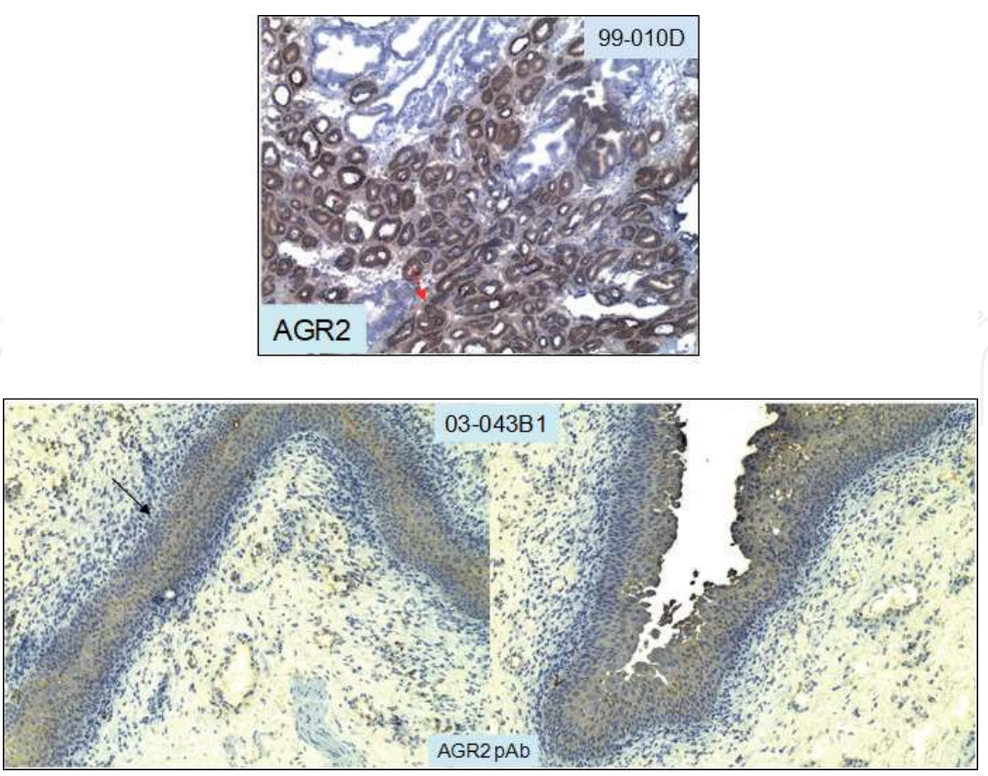

Figure 1.
AGR2 in prostate cancer and bladder. The prostate tumor glands (specimen 99-010D, top) are stained positive for AGR2 while benign glands are negative. Faint staining in the cancer-associated stroma (red arrow) suggests AGR2 secretion from the adjacent cancer cells. The urothelium (specimen 03-043B1, bottom) is stained at moderate intensity. Two different parts of the specimen indicate uniform expression throughout the entire urothelium. Black arrow indicates the lamina propria below the urothelium.

by DNA microarray analysis of $CD9^+$ urothelial cells (signal value = 105.54) [25] and $CD26^+$ prostate cancer cells (signal value = 4168.03) [10]. A previous study estimated that adenocarcinoma cells had 80% iAGR2 and 20% eAGR2 while in all non-tumor cells AGR2 was located intracellularly [19]. Faint AGR2 staining of the stroma surrounding prostate tumor glands could be evidence of AGR2 secretion [27] (**Figure 1**, top panel). No such staining was detectable in the lamina propria next to the $iAGR2^+$ urothelium [18] (**Figure 1**, bottom panel) or benign glands of the prostate. In prostate cancer cells, AGR2 could be activated by ER stress with down-regulation of unfolded protein response genes leading to an accumulation of misfolded proteins like that in pancreatic cancers cells [28].

In summary, three AGR2 expression patterns have been described in solid tumors: (1) iAGR2 positive for normal urothelial cells, eAGR2 positive for 25% primary tumors [18]; (2) no AGR2 for prostate epithelial cells, eAGR2 positive for >95% primary tumors [10], similar to pancreas [12] and breast [13]; (3) iAGR2 positive for normal bronchial epithelial cells, eAGR2 positive for>95% non-small cell lung tumors [14].

3. Differential subcellular localization of AGR2

The contrasting localization of eAGR2 and iAGR2 is well illustrated by the bladder. The entire urothelium is positive for AGR2 expression as shown by immunostaining [18] (**Figure 1**). If AGR2 is secreted, then a substantial amount of this protein would be found in voided urine. In fact, little AGR2 is released by normal $iAGR2^+$ urothelial cells as detected by ELISA [18]. This result was supported by urine proteome database queries that AGR2 was not listed in the *UrinePA-PeptideAtlas* of 2,500 proteins profiled by large-scale proteomics [29], and in the core urinary proteome of 587 proteins obtained from healthy people [30]. In contrast, we found that $AGR2^+$ bladder cancer cells secreted AGR2. Urine from a bladder cancer patient was scored 7.5-fold higher than buffer control for AGR2. Five of 20 bladder cancer patient urine in one cohort were tested positive (AUC = 0.75), which matched the 25% bladder cancer being positive for AGR2 expression [18]. Our urine assay also detected AGR2 secreted by prostate cancer producing a similar AUC [31]. For serum, level of AGR2 is near background in healthy people [32]. Query of the *PeptideAtlas* database yielded very low AGR2 signature peptide counts. However, when sera of five prostate cancer patients were analyzed for AGR2, there was a strong correlation, $R^2 = 0.93$, found between levels of AGR2 (in pg/ml) and those of PSA (in ng/ml) [32]. This result would not be possible if there was a basal level of blood AGR2 arising from possible secretion by $AGR2^+$ cells of normal tissue such as the urothelium and lung epithelium into the circulatory system [32].

4. Differential expression of AGR2 in prostate cancer – primary vs. metastasis

AGR2 in prostate cancer shows two seemingly conflicting features in tumor biology. High expression in primary tumors is linked to better patient survival. Together with cell surface CD10 (membrane metallo-endopeptidase), four cancer phenotypes can be distinguished: $AGR2^+CD10^-$, $AGR2^-CD10^+$, $AGR2^-CD10^-$, and $AGR2^+CD10^+$; normal luminal cells are $AGR2^-CD10^+$. For high-grade disease, the $AGR2^+CD10^-$ phenotype is associated with a near 10-fold survival advantage than that of $AGR2^-CD10^+$; those of $AGR2^-CD10^-$ and $AGR2^+CD10^+$ in between [27]. Not unexpectedly, a majority of these tumors were typed $AGR2^-CD10^+$ (**Figure 2**). It appears that CD10 plays an important role in the extraprostatic

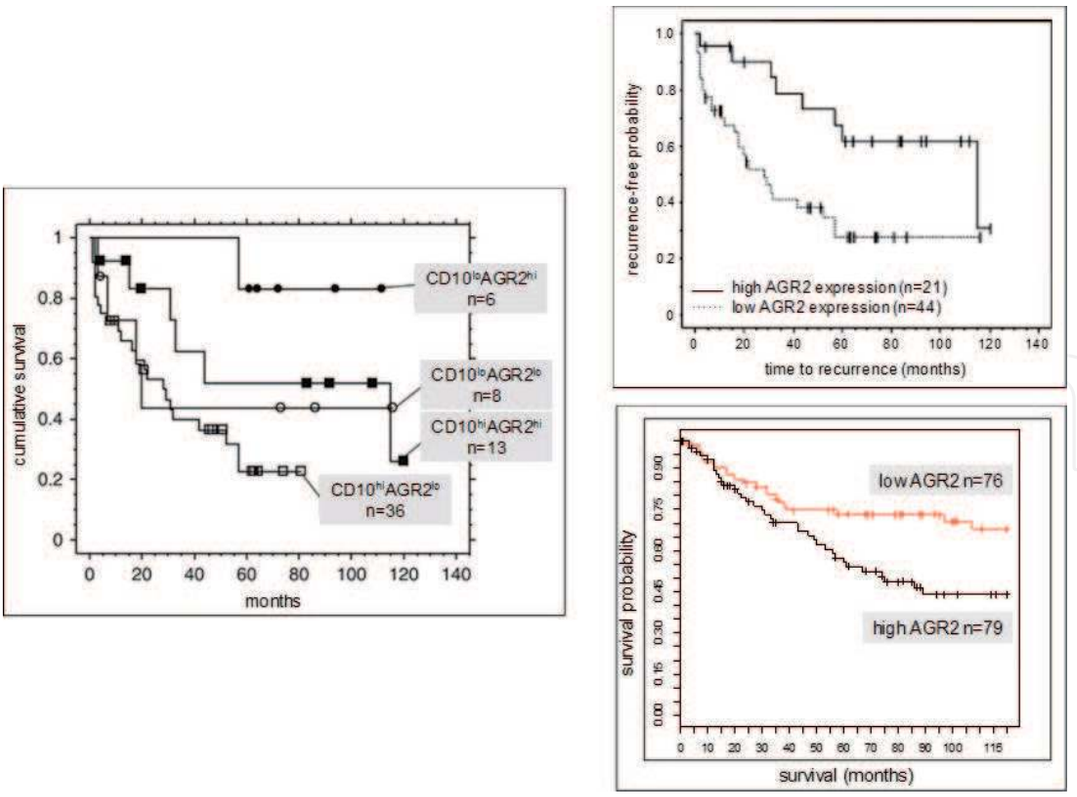

Figure 2.
AGR2 expression and patient survival. Left: the plot shows the outcome of high-stage patients grouped by AGR2 and CD10. At 5 years, 85% of AGR2^{hi}CD10^{lo} were recurrence-free compared with just 25% of AGR2^{lo}CD10^{hi}. Right: survival analyses for prostate cancer (top) and lung cancer (bottom) patients (<65y) show a contrasting trend.

spread of cancer cells to local lymph nodes because cancer cells in involved regional lymph nodes are invariably CD10^{+}(AGR2$^{-/lo}$) [33]. Node metastasis-derived cancer cell line LNCaP and patient-derived xenograft (PDX) LuCaP 35 are CD10^{+}AGR2^{-} and CD10^{+}AGR2^{lo}, respectively. A notable feature is the localization of CD10 to cell interior in cancer cells of higher grades where it appears to interact with cytosolic heat shock proteins [34]. As in the case for AGR2, CD10 has extracellular eCD10 and intracellular iCD10, but in this case, iCD10 is specific to cancer cells. This suggests that protein trafficking is abnormal in cancer cells. AGR2 expression is also associated with prostate cancer differentiation, with Gleason grade 3 (well-differentiated) cancer cells showing a 10-fold higher level than Gleason grade 4 (less differentiated) cancer cells [10]. Its association with lower tumor grade predicts better survival for patients harboring AGR2^{+} tumors [11] (**Figure 2**). Similarly, in breast cancer AGR2 is associated with better survival [35]. In contrast to AGR2, CD10 is more prominent in higher grade prostate tumors, and is associated with poorer survival for patients [33, 36].

However, most of the distal bone and soft tissue metastases contain cancer cells with the AGR2^{+}CD10^{-} phenotype, the exception being tumors of small cell carcinoma (AGR2^{-}CD10^{-}) [27], (**Figure 3**). PDX LuCaP lines established from samplings of these metastases are also AGR2^{+} [27]. These results underscore the important role of AGR2 in cancer spread as we reported for pancreatic cancer [37]. Possible mechanisms on how AGR2 promotes metastasis include disruption of epithelial cell adhesion, imparting invasive behavior to tumor cells [19], inducing apoptosis in susceptible normal cells by secreted AGR2 to undergo apoptosis [38], and through activating matrix metalloproteases, cathepsins B and D [37], which could facilitate tumor cells access to the circulatory system. Importantly, inhibition of AGR2 expression in lung cancer cells leads to their loss of metastatic capability [19].

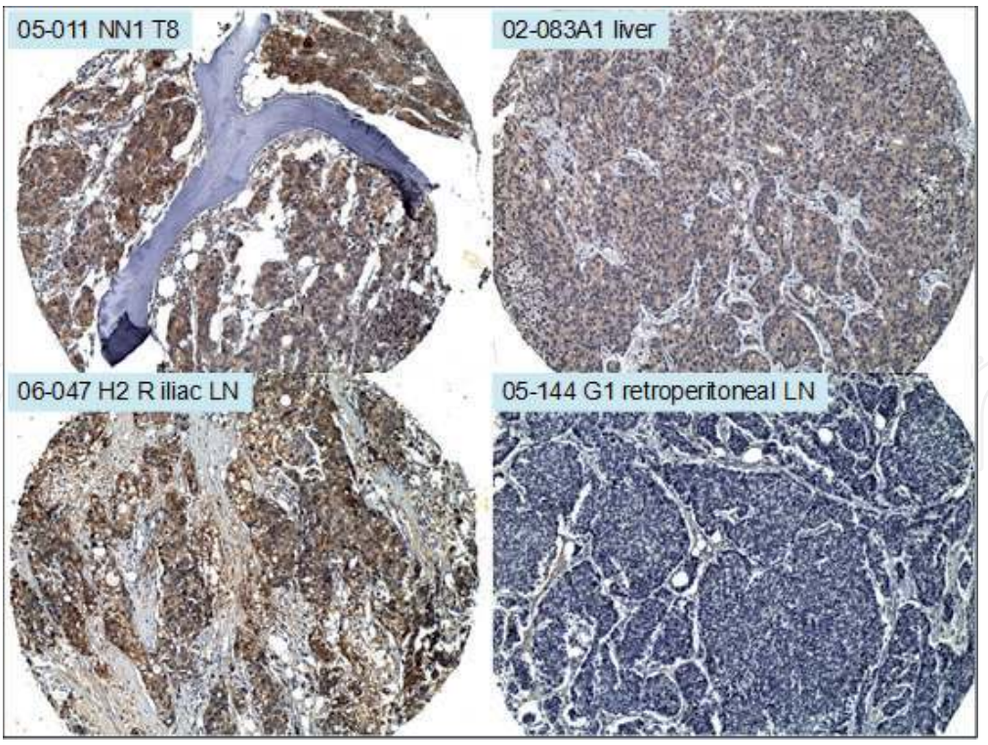

Figure 3.
AGR2 in prostate cancer metastases. Tumor cells in distal metastases – bone (top left), liver (top right), lymph node (bottom left) – are strongly stained for AGR2. A metastasis containing small cell carcinoma (bottom right) is not stained.

5. AGR2 in bladder cancer and lung cancer

Although 25% primary bladder tumors of a study cohort were found positive for AGR2, this percentage increased to 45% in the synchronous lymph node metastases [18]. The discordance between primary cancer and lymph node metastasis could involve a phenotypic change [39], which could occur in bladder cancer cells going from $AGR2^-$ to $AGR2^+$. AGR2 in bladder cancer, unlike prostate cancer, showed no link to patient survival in one cohort analyzed [18]. A role for CD10 in lymph node spread of bladder cancer was also not apparent [18]. Recently, UW Urology initiated the bladder cancer rapid autopsy program modeled on the Department's success in obtaining>40 prostate cancer LuCaP lines with various characteristics [40], and has established lines, dubbed CoCaB, from primary and metastatic urothelial cancer. To date, seven lines are available: CoCaB 1, CoCaB 8 urothelial carcinoma, CoCaB 11, CoCaB 19 squamous carcinoma, CoCaB 12 urothelial with sarcomatoid component; three lines from metastases of deceased patients: CoCaB 10 (liver metastasis) urothelial carcinoma, CoCaB 14.1 (omentum metastasis) and CoCaB 14.2 (liver metastasis) urothelial carcinoma with squamous features. These lines were profiled by RNAseq and exome sequencing. Notably, the lines derived from metastases showed high AGR2 expression (**Figure 4**). This is in line with our result on prostate cancer metastases where, except for small cell carcinoma, all tumors as well as the LuCaP lines derived from metastases showed high AGR2 expression [27]. Likewise, pancreatic cancer metastases invariably showed high AGR2 expression [37].

In non-small cell lung cancer, higher AGR2 expression in primary tumors is associated with a poorer outcome for patients under 65 [14, 19], though over 90% of the tumors are $AGR2^+$ (**Figure 2**). This is in contrast to the finding for prostate cancer. Thus, depending on the microenvironment, the metastatic function of AGR2 is not predominant in differentiated prostate tumors. When cancer cells have escaped the prostatic capsule (via CD10), AGR2 appears to be essential for distal

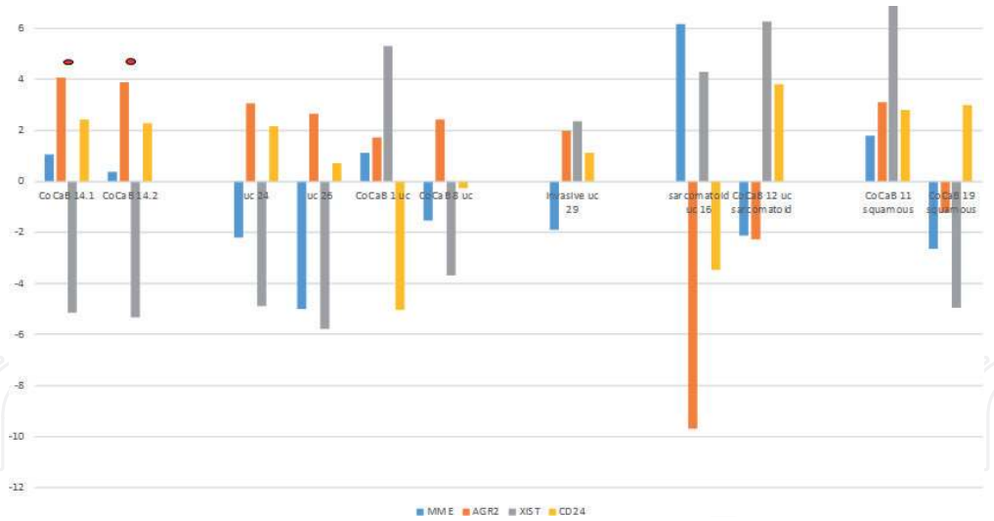

Figure 4.
Bladder cancer cell types. Cancer cell lines are identified on the x-axis (CoCaB and uc). Histogram represents fold in expression difference as scored by RNAseq signal values on the y-axis for MME/CD10, AGR2, XIST (X-inactive specific transcript) and CD24. The two CoCaB lines established from metastases are indicated by small red oval.

colonization of other organs for prostate cancer cells. In the disease course, AGR2 expression could be down- or up-regulated in cancer cells through mechanisms yet to be elucidated.

A meta-analysis of the published data on the clinical utility of AGR2 expression in various solid tumors summarizes the link to survival findings [41]. AGR2 expression in primary cancer can be associated with better survival for prostate cancer, no survival advantage in bladder cancer, poorer survival for lung cancer. While AGR2 in pancreatic cancer could have a significant impact in treating both early and advanced diseases, anti-AGR2 therapy in prostate cancer could prove promising in treating advanced disease as 96.4% metastatic lesions of adenocarcinoma being $AGR2^{+}$ against 0.7% of $AGR2^{-}$ small cell carcinoma and 2.9% of mixed carcinoma type [42].

6. Chimeric human:mouse AGR2 antibodies

Mouse monoclonal antibodies to AGR2 were generated by inoculating bacterially produced recombinant AGR2 in RBF/DnJ mice [20]. Two of eleven clones collected, P1G4 (mIgG1) and P3A5 (mIgG2a), were tested positive for binding to native AGR2. These antibodies perform well in immunostaining of frozen tissue sections but not formalin-fixed tissue. ELISA based on these two antibodies was able to detect 17 pg of cancer-secreted AGR2 in 100 μg of total urinary protein isolated from a patient diagnosed with a tumor of 5.5 cc in size [20].

Based on our published design [43], the mouse antibodies were converted to chimeric human:mouse. The mouse variable V_H and V_κ of P3A5 and P1G4 sequences were determined via reverse transcriptase-polymerase chain reaction (RT-PCR) with designed primers from mIg mRNA isolated from the respective hybridoma cell lines. The V sequences were-matched to known murine Ighv and Igkv genes. The human constant C_γ and C_κ domain cDNA were cloned by using designed primers from white blood cells of healthy donors. The hIg cDNA were verified by DNA sequencing and restriction enzyme digestion. The C_γ (digested by *Apa* I and *Bam* HI) and C_κ (*Hind* III and *Avr* II) modules were joined to the V_H (*Eco* RV and *Apa* I) and V_κ (*Bam* HI and *Hind* III) modules, respectively, in plasmid vector pVITRO1*neo*. Each cDNA contained a Kozak box. Plasmids of chimeric P1G4 hIgG1, hIgG2,

hIgG4; P3A5 hIgG1, hIgG2, hIgG4 were generated [22]. The different Cγ plasmids could be distinguished by *Sac* II and *Eco* RI digestion. Cγ3 sequences were not detected in the cDNA prepared from 1 ml of blood.

Human embryonic kidney fibroblasts, HEK293F, were transfected by the hIgG plasmids, selected for G418 (neomycin) resistance and cloned. RT-PCR analysis of the transfected cells showed equivalent mRNA levels for the 560 bp neo, 720 bp IgL and 1420 bp IgH [22]. The culture media supernatant was assayed for AGR2 binding. P1G4 was used to capture AGR2 (secreted by eAGR2$^+$ prostate cancer LuCaP 147 in tissue collagenase digestion media to obtain single cells from minced tumor pieces) followed by P3A5 (positive control) and culture supernatant containing the chimeric antibodies. HRP-conjugated anti-mIgG2a or anti-hIgG were used for detection. The chimeric antibodies were found similar to P3A5 in AGR2 binding [22]. Untransfected 293F cells or transfected with a defective L-chain construct produced no binding. Media from serial culture passages showed that hIgG synthesis continued indicating stable integration of the transgenes into the host genome. The hIgG-producing clones were weaned from fetal bovine serum supplement, and cultured in the absence of drug for G418 is toxic. The serum-free growth media contained few other proteins (293F cells are non-secretory fibroblasts compared to hybridoma cells), and a spin-filtration step using a 30 K molecular weight cut-off could readily concentrate the 150 kDa IgG proteins [22]. If necessary, each antibody can be purified further on protein G-sepharose. The chimeric hIgG1, hIgG2, hIgG4 and mIgG2a P3A5 detected similar amounts of AGR2 secreted by different LuCaP lines: 147, 35CR, 86.2, 105 (**Figure 5**). Thus, these chimeric antibodies retain specific antigen binding and can be produced more economically via large-scale cell culture (instead that of hybridoma cells, which also produce a defective non-specific mouse light chain, and potentially infectious murine bioactive agents).

To increase the production of hIgG by transfected 293F cells, we transferred the different hIgG gene cassettes into plasmid vector pVITRO1*bsr* (encoding blasticidin resistance). Selected hIgG-producing neo^R clones were transfected by the *bsr* constructs and selected for blasticidin resistance. The resultant neo^{R}bsr^R clones showed increased amounts of secreted hIgG in the culture media due to the increased gene dosage [22].

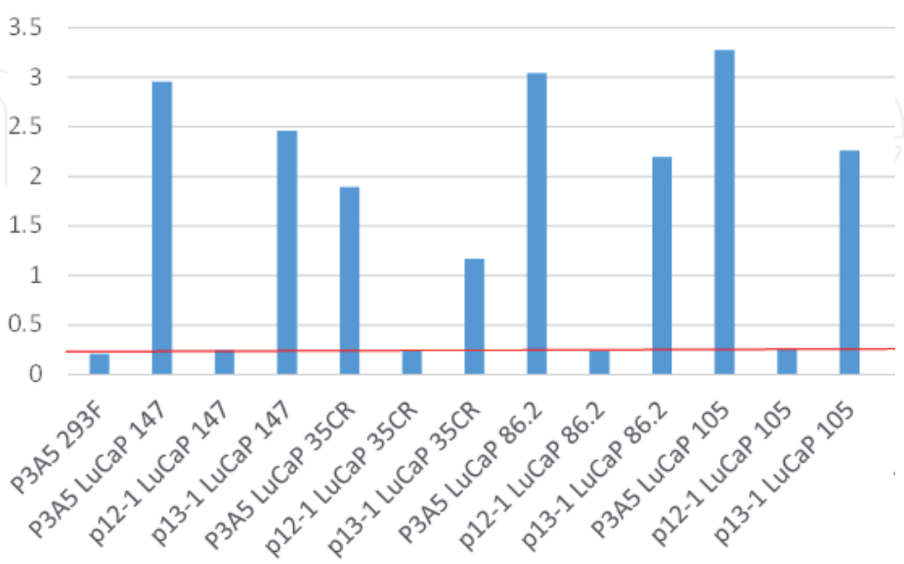

Figure 5.
Chimeric IgG binding to AGR2. Both chimeric (transfected HEK clone p13–1) and P3A5 detect varying amounts of AGR2 secreted by different LuCaP PDX lines. Clone p12–1 is a defective construct that produced untranslated L chain mRNA. Similar binding was also shown by the other IgG types. The absorbance units of ELISA are on the y-axis.

7. Cancer cell surface expression of eAGR2 and tumor localization

To demonstrate cancer cell surface expression, we used flow cytometry with our obtained monoclonal antibodies. The mouse Agr2$^+$ pancreatic cell line DT6606 [44], derived from an engineered C57BL/6 mouse strain to develop pancreatic cancer [45], was incubated with P3A5 followed by dye-conjugated anti-mIgG2a. Antibody binding to the cell surface was indicated by fluorescence shift (*vs*. isotype-matched control) [22]. Most available anti-human AGR2 antibodies, like P3A5, recognize both human AGR2 and murine Agr2 as the two proteins share a high degree of sequence homology. Human pancreatic cells were previously shown to have cell surface expression of eAGR2 using a different antibody [37].

To demonstrate tumor localization, ^{111}In-radiolabeled P3A5 was injected into mice carrying DT6606 tumors. At 48 h post-injection, the implanted tumors showed intense labeling (**Figure 6**). There was minimal labeling of the iAgr2$^+$ bladder or lung, or elsewhere [22]. The imaging confirmed cancer cell surface localization as well as cancer cell specificity of eAgr2. The cross-reactivity between human AGR2 and mouse Agr2 allowed one to speculate that a similar result would be obtained in human patients, i.e., specific localization to eAGR2$^+$ pancreatic (or other solid) tumors and not to iAGR2$^+$ organs. With cancer-specific localization, there is a strong likelihood that ant-AGR2 would be highly effective against cancer with minimal reactivity towards non-cancer tissue.

8. Enhancement of chemodrug inhibition of tumor by antibody

Our P1G4 antibody was found to possess a clinically useful property. Using eAGR2$^+$ pancreatic PDX in mice, the combination of pancreatic cancer drug Gemcitabine (Gem) and P1G4 (P1) reduced tumor growth compared to Gem alone. This difference was statistically significant ($P < 0.05$) [22]. Immunostaining for Ki67 indicated less (AGR2$^+$) tumor cell proliferation in P1 + Gem, which was manifested by the size of the corresponding resected tumors (**Figure 7**). ELISA indicated that serum AGR2 levels could be correlated with tumor burden. Once Gem was discontinued, the tumors in the Gem-only group relapsed immediately, and grew at a faster rate than those in the P1 + Gem group despite an effectively reduced antibody concentration as antibody treatment was terminated after 28 days. The combination of P3A5 (P3) and Gem showed no such enhancement. Parenthetically, our data also confirmed that Agr2 was not secreted from normal mouse organs into blood. The

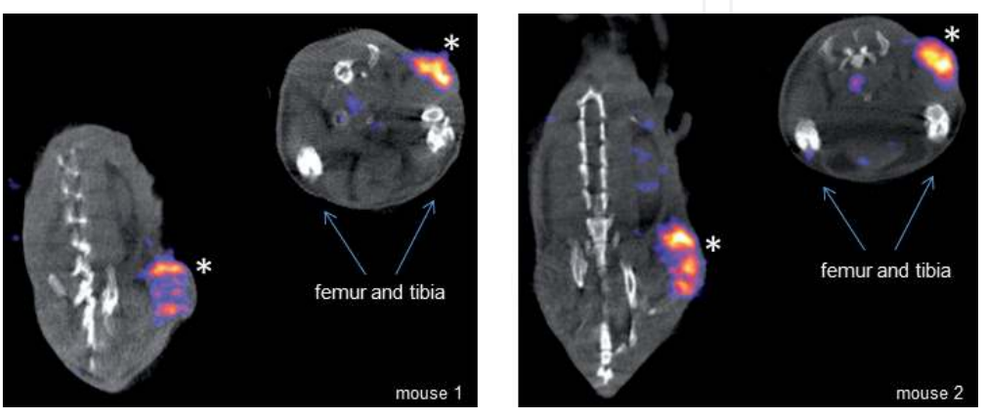

Figure 6.
Tumor localization of P3A5. The SPECT/CT scans show specific uptake of radiolabeled P3A5 by Agr2$^+$ DT606 tumors (*) in two C57BL/6 mice.

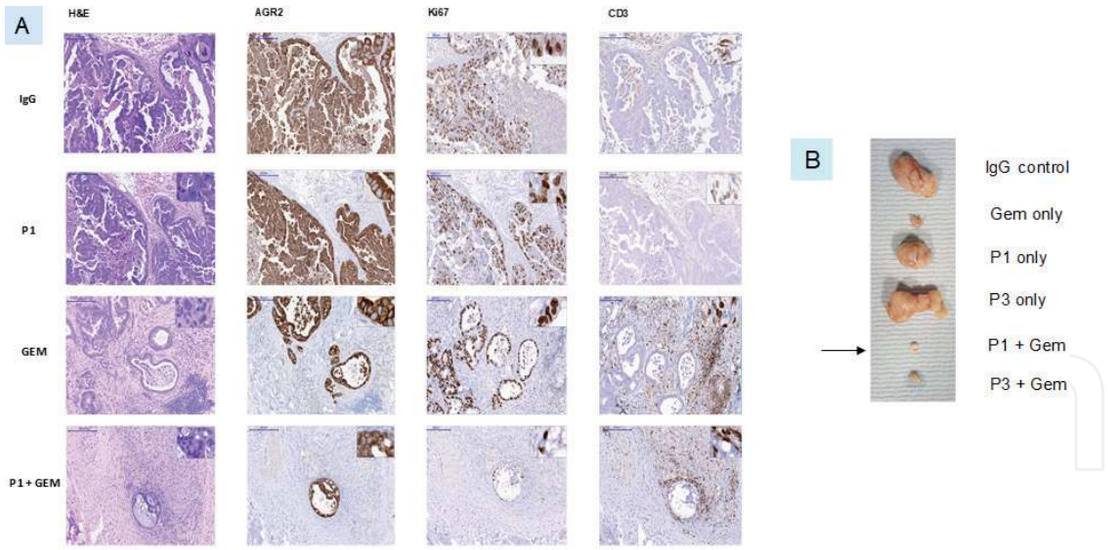

Figure 7.
Drug inhibition of tumor growth enhancement by P1G4. (A) Representative immunohistochemistry images show the effect (from top to bottom) of IgG control, P1G4 (P1) alone, Gemcitabine (GEM) alone, P1 + GEM. Ki67 staining indicates that tumors treated with drug still had high proliferation rate, which was limited in P1 + GEM tumors. CD3 shows T cell infiltration in the GEM and P1 + GEM groups. (B) Tumors resected at week 6 from the different treatment groups are compared. The smallest size is found in the P1 + GEM group (arrowed).

internal organs of liver, spleen, stomach, intestine, colon, and pancreas were histologically examined, and no visible pathologic changes were identified. The sparing of organs in anti-AGR2 tumor targeting was also reported by another group [46]. The mechanism behind this epitope-dependent phenomenon is unknown but could be related to a reported observation of increased tumor inhibition by an antibody to AGR3 in combination with the chemodrug cisplatin [47]. AGR3 is a close family member of AGR2. Both AGR2 and AGR3 tend to be elevated in cancer, though to different levels as found in prostate cancer [10]. The combination of monoclonal antibody plus biological inhibitors are being pursued to treat more successfully non-small cell lung cancer [48].

9. Tumor cell lysis in vitro

In our earlier work, ^{51}Cr radiolabeled target cancer cells were exposed to TAA antibodies and human serum (as a source of complement factors) or peripheral blood leukocytes [43, 49]. By CDC, the chimeric antibody generated higher cytotoxicity at all complement dilutions. By ADCC, at a ratio of 100:1 blood leukocytes to target cells, the chimeric lysed a greater fraction of the cancer cells and gave 50% cytolysis at 100-fold lower concentration than the mouse antibody. ADCC was observed at a 3:1 ratio of effector to target cells when the chimeric (at 2.5 μg/ml) was used. Cell killing was specific because ADCC was not observed with cell lines lacking the target antigen.

To test the anti-tumor effect of chimeric antibodies, we incubated PC3 prostate cancer cell line in the presence of donated human serum. Like pancreatic cancer cells, cell surface expression of eAGR2 was found on PC3 cells. Spin-concentrated chimeric IgG was used with human serum for CDC. PC3 cells were incubated with freshly donated human serum and added AGR2 antibodies. There was no observable effect on cell viability in the culture well with human serum only, as was the well with mouse P3A5 + serum. In the well with a cocktail of chimeric IgG1, IgG2, and IgG4, cell growth was inhibited with well surface showing areas devoid of cells,

and clusters of pyknotic cells in suspension [22]. We postulate that a combination of IgG subtypes would be more effective than IgG1 alone since our normal immune response produces these IgG types, each exhibiting a unique profile with respect to immune complex formation, complement activation, recruitment of effector cells, and half-life [50]. For example, strong antitumor activities were observed for an IgG3 antibody targeting a melanoma-associated ganglioside [51]. We did not obtain chimeric IgG3 for our monoclonal antibodies. We will attempt to clone $C_\gamma 3$ from a larger volume of blood or a pool of several donations using γ3-specific primer oligonucleotides. Our cloned human C_γ and C_κ can accept any new V_H and V_L of antibodies developed against novel TAA.

10. Antibody-drug conjugation

In addition to antibodies that rely on immune system components, a cytotoxic drug can be linked directly to the AGR2 antibodies to produce an antibody-drug conjugate (ADC). ADC delivers the drug payload to the target organs or tissues [52]. For anti-AGR2 ADC, drug compounds were constructed by covalently linking poly(N-isopropylacrylamide) to both P1G4 and P3A5 via carbodiimide chemistry. The linking polymer was synthesized by reversible addition fragmentation chain transfer (RAFT) with a carboxylate chain end [53]. To conjugate antibodies, the carboxylate was converted to an active ester for formation of an amide bond to lysine residues [53–56]. We have also developed a block copolymer with tetrafluorophenyl (TFP) ester monomers to drive the antibody conjugation [57]. The resulting anti-AGR2 ADC were confirmed by gel electrophoresis, which showed the larger molecular weight products compared to the unconjugated antibodies (**Figure 8**). After the size-exclusion chromatography, the purified ADC were shown by ELISA to bind AGR2 (**Figure 8**). Sample solutions containing a constant AGR2 concentration were mixed with ADC from 125 to 1,000 ng/ml. ELISA measured the unbound AGR2 with higher ADC concentrations resulting in less free AGR2 in the solution. Thus, our conjugation chemistry did not affect appreciably the antigen binding affinity of the antibodies. Polymer chains with improved loading capacity by incorporating functional groups [58] could be developed for conjugation to docetaxel, doxorubicin, and other drugs. To improve delivery efficiency, the polymer can be engineered to increase circulation time, and the polymer composition can be modulated.

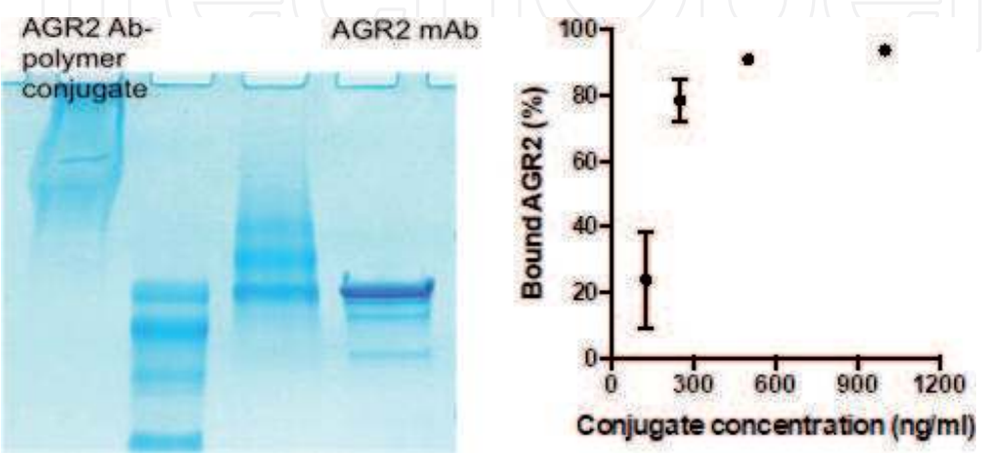

Figure 8.
Antibody drug conjugate. The left panel shows the conjugated products (lane 1) vs. unconjugated antibody (lane 4). The right plot shows more AGR2 bound (percentage, y-axis) with higher ADC concentrations of the conjugate (in ng/ml, x-axis).

11. Adaptation of PDX LuCaP lines to in vitro culture

For therapeutic testing, the LuCaP series of >40 different models established from patient tumor samples donated at autopsies and surgeries provide an invaluable resource than long-used cultured cell lines. They have been molecularly and pharmacologically characterized, and encompass a large spectrum of the disease course and representative of human prostate cancer [39]. Transcriptomics and genomics data have shown that the gene expression of these cancer cells was concordant with that of the human tumors from which they originated. Indeed, these models were used in the preclinical study to determine efficacy of anti-prostate-specific membrane antigen (PSMA) ADC as they show a range of PSMA expression [59]. For AGR2, concordant expression has been determined by DNA microarrays [40], immunostaining [27], and ELISA measurement of secreted AGR2 [20, 38]. Similar to prostate cancer patient specimens, the adenocarcinoma lines are positive for AGR2 while the small cell carcinoma including some non-adenocarcinoma are negative or low for AGR2.

Their utility could be increased if they can be grown outside the mouse for in vitro testing of ADCC and CDC. We showed that LuCaP cells prepared from freshly excised tumors could be successfully cultured long-term in the presence of irradiated mouse embryonic fibroblasts (MEF) as feeder [60]. Furthermore, LuCaP cells could be viably frozen using a protocol for stem cells [60], which makes constant harvests from animals unnecessary. Both adenocarcinoma and small cell carcinoma LuCaP lines could be thus grown in culture (unpublished data). The in vitro-adapted cell lines with differential AGR2 expression could be used to determine the molecular mechanism controlling AGR2 expression in cancer cells. The same methodology can be used to adapt CoCaB cells for in vitro growth and testing.

12. Future work and developments

12.1 Preclinical testing of chimeric antibodies

With the availability of chimeric antibodies and ADC, we can carry out animal studies to assess safety and efficacy of anti-AGR2 therapy, especially in PDX models available in prostate and bladder cancers. The LuCaP lines recapitulate the molecular heterogeneity of metastatic castration-resistant prostate cancer. Overall, a majority of molecular events characterized in human prostate tumors are found in LuCaP such as *AR* amplification, *PTEN* loss, *RB1* deletion, DNA damage response deficiencies [40, 61]. These LuCaP lines can be selected for study: $AGR2^+$ LuCaP 23.1, 35, 136, 147 and $AGR2^-$ LuCaP 145.1 to test P1G4-ADC, P3A5-ADC, P1G4 + docetaxel, P3A5 + docetaxel. With regard to AGR2 expression, LuCaP 23.1 and LuCaP 147 have relatively high levels, while small cell carcinoma LuCaP 145.1 as control has no expression [27]. Complete effect from anti-AGR2 would be expected for the former examples and no effect for the latter. For IgG control, we can transfect 293F cells with a construct containing H chain of P3A5 and L chain of P1G4 or H chain of P1G4 and L chain of P3A5, which would be expected to produce an AGR2 non-binding V domain. Green fluorescence protein (GFP)-labeled chimeric antibodies could be used for tumor localization (to show no labeling of $iAgr2^+$ normal organs) in place of radioisotopes, if necessary, to add to our pancreatic cancer PDX result. The study will also test if P1G4 has an enhancement effect in tumor growth inhibition by docetaxel as shown by this antibody in the Gemcitabine study. Response by LuCaP lines to docetaxel treatment varied substantially [40]. A high dose (20 mg/kg) generally produced growth suppression and survival benefits.

Only LuCaP 86.2 showed response at a low dose. Others like LuCaP 35 showed reduced survival as monitored by body weight loss despite tumor growth inhibition. We will test whether the response to docetaxel will be less variable and more pronounced with the addition of P1G4 (but not P3A5). The P1G4-docetaxel ADC may act in an equivalent way as P1G4 + docetaxel. Docetaxel was the first chemotherapeutic drug shown to prolong patient survival, and is in widespread use [62].

The different anti-AGR2-ADC are tested through intraperitoneal injection twice a week for four weeks. In our pancreatic cancer study, a 5 mg/kg antibody concentration was shown to be effective in the P1G4 + Gemcitabine arm. LuCaP tumor volume and body weight are monitored, and treatment will last four weeks. Tumors are collected at study end for histology, and the internal organs are examined for anti-Agr2 effect, if any. Serum is collected for AGR2 measurements by ELISA. Serum PSA may also be measured to see if there is concordance between these two biomarkers. Other parameters to try include higher antibody concentration, longer treatment time. The in vitro adapted LuCaP cells will allow us to determine the effective dose of serum and effector cell donations from multiple individuals (and if available, blood from cancer patients whose immune response may be compromised by their disease) in CDC and ADCC, respectively, mediated by the chimeric IgG. The optimal antibody concentration (μg/ml) for 10^6 cancer cells is determined. It is possible that a higher dose is required since AGR2 is secreted by the tumor cells where a portion of the antibodies might be bound to the free antigen. This in vitro assay is in effect an immune system model for the human body. PDX lines can also be grown as spheroids/organoids in vitro [63]. Organoids could be used to represent a solid tumor mass *vs*. monolayer culture to test the efficacy of chimeric antibodies.

In the past years, an ADC to PSMA was shown to produce clinically relevant decline in serum PSA and circulating tumor cell counts in metastatic castration resistant, taxane-experienced and chemo-naived prostate cancer patients [64]. At a working dose of 2.3 mg/kg, side effects reported in some patients include neutropenia, fatigue, electrolyte imbalance, anemia and neuropathy. A small number died from disease progression. PSMA is a membrane metalloenzyme (FOLH1) found in the kidney, small intestine, central and peripheral nervous systems [65]. Non-exclusivity of this TAA to prostate cancer likely accounts for the range of side effects observed. The co-targeting of normal cells has led to recall of, for example, gemtuzumab because of severe complications since the TAA, CD30, is present on both leukemic blast and normal cells [66]. Importantly, not all prostate cancer cells express PSMA. Anti-PSMA-MMAE (monomethyl auristatin E) was shown less or not effective against tumors with low or null PSMA expression [59]. In this efficacy study using LuCaP cells, complete tumor regression for LuCaP 96CR with the highest PSMA expression was found, and no response for LuCaP 58 with the lowest PSMA expression [59]. However, intermediate effect was found for LuCaP 77 and LuCaP 105, two lines with similar PSMA expression levels as LuCaP 96CR. Why the difference was not explained. Thus, the inherent pitfall in prescribing anti-PSMA therapy by itself is selection of PSMA-negative tumors. Nonetheless, this PSMA ADC is currently under large clinical trials [64, 67].

The rationale of our developing a second prostate cancer immunoreagent is that anti-eAGR2 could complement anti-PSMA when used in combination, which could be effective against $PSMA^-AGR2^+$, $PSMA^+AGR2^-$, and $PSMA^+AGR2^+$ cancer cells. Note LuCaP 35 is negative for PSMA. This particular model is used to show whether anti-AGR2 will inhibit its growth where anti-PSMA could not. Two other attributes additionally bolster our rationale. eAGR2, unlike PSMA, is tumor-specific as normal cells do not express eAGR2, and metastatic prostate cancer cells express high levels of eAGR2. The two AGR2 epitopes (P1G4 and P3A5) targeted could also ensure that allelic changes in one of the epitopes will not affect susceptibility of the

cancer to anti-AGR2. For bladder cancer, we will carry out a similar study employing the AGR2$^+$ CoCaB lines to test the ADC and P1G4 + cisplatin. Cisplatin is more commonly used to treat this cancer, sometimes in combination with Gemcitabine [68]. In vitro-adapted CoCaB lines are similarly used for CDC and ADCC testing.

Cancer treatment by antibody is well established and proven to be effective. One good example is trastuzumab to target HER2/EGFR (CD340) for a subset of breast cancer and non-small cell lung cancer [69, 70]. The major obstacle to more success in antibody therapy is the time-consuming need to discover TAA for each type of cancer that at a minimum is expressed by only a few normal cell types. eAGR2 is not only expressed in prostate cancer cells but also multiple tumor types so that anti-AGR2 would have a much wider application than just treating one or two cancers. Our experiments will demonstrate the validity of this claim.

12.2 Lineage relationship between AGR2$^+$ and AGR2$^-$ prostate cancer

The use of anti-AGR2 might lead to the selection of AGR2$^{lo/-}$ non-adenocarcinoma cancer including small cell carcinoma. The introduction of newer anti-androgen therapies of late has led to an almost 20% patients presenting small cell carcinoma at treatment failure [71]. Of critical importance is finding a means to prevent the emergence of AGR2$^-$ tumors.

Figure 9 shows our model of prostate cancer differentiation relating luminal-like AGR2$^+$ adenocarcinoma to AGR2$^-$ more stem-like small cell carcinoma [72]. Stem-like cancer cells could arise from de-differentiation of luminal-like cancer cells. This process is akin to reprogramming of somatic cells via forced expression of a set of stem cell transcription factors (scTF) to induced pluripotent stem (iPS) cells [73]. We demonstrated that prostate adenocarcinoma cells could be so reprogrammed to stem-like, small cell carcinoma-like derivatives with scTF LIN28A, NANOG, POU5F1, SOX2 [60]. A relevant clinical finding is that tumors with Gleason score ≥ 8, i.e., less differentiated, tended to show a shorter interval to the emergence of small cell carcinoma [74], as the non-glandular tumor cells are closer to stem-like in lineage. On the other hand, stem-like cancer cells could be induced to differentiate into luminal-like cancer cells by prostate stromal mesenchyme cell factors [75]. From early tissue recombinant studies, stromal cells were found to determine the specificity of urologic organ development [76, 77]. Thus, prostate stromal cells would induce stem/progenitor cells, regardless of tissue origin, to differentiate into prostate; bladder stromal cells into bladder. This induction involves secreted factors and heterotypic cell contact. We identified proenkephalin (PENK, 267 aa) and stanniocalcin 1 (STC1, 247 aa) as prostate stromal-specific genes encoding

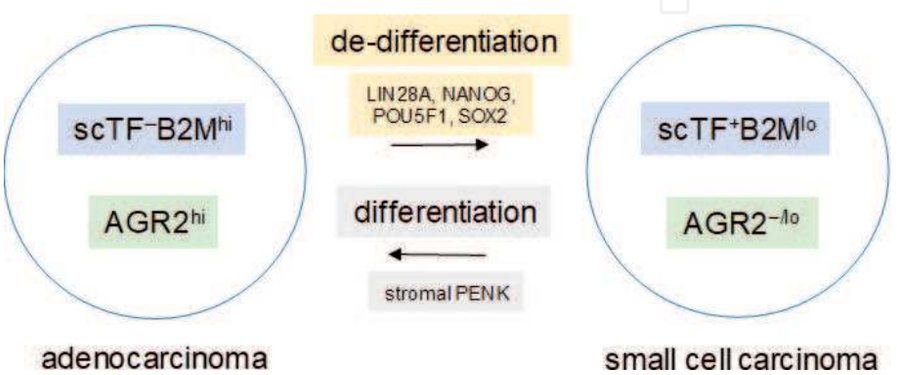

Figure 9.
Lineage of prostate cancer cell types. Luminal-like, non-stem-like adenocarcinoma AGR2^{hi}, scTF$^-$B2M^{hi} and stem-like AGR2$^{-/lo}$, scTF$^+$B2M^{lo} small cell carcinoma are related by de-differentiation with activation of scTF. The reverse of the process can be triggered by stromal cell factors such as PENK.

secreted hormones [78]. When cultured stem-like cells were incubated with prostate stromal cell conditioned media (containing stromal secreted molecules) PENK and STC1 were specifically induced in the differentiating stem cells. The resultant cells showed a change in colony morphology as well as one in transcriptome [79]. PENK was not induced by $PENK^-$ bladder stromal cell or $PENK^-$ prostate cancer-associated stromal cell conditioned media [79, 80]. The absence of PENK in the stroma of tumor foci suggests that it could be an underlying contributing cause of cancer development.

PENK cDNA (803 bp) was cloned from microdissected benign prostate tissue [10], and was transfected into small cell carcinoma LuCaP 145.1 cells. LuCaP 145.1 is stem-like because of its expression of scTF, which are, as a quartet, absent in non-stem-like LuCaP adenocarcinoma lines [60, 81]. In addition, LuCaP 145.1 was found to share expression of other genes with stem cells, including the down-regulation of β2-microglobulin (B2M) [60, 81, 82]. Forced expression of PENK in LuCaP 145.1 down-regulated the scTF, and up-regulated in tandem B2M [75]. Thus, a phenotypic change of stem-likeness ($scTF^+B2M^{lo}$) exhibited by LuCaP 145.1 to a phenotype ($scTF^{lo/-}B2M^{hi}$) more characteristic of differentiated cancer cells was produced by PENK. In other words, PENK can counteract the activity of scTF. Therefore, PENK and other stromal factors could be envisioned as effective agents in differentiation therapy to maintain adenocarcinoma in the differentiated state with high AGR2 expression.

The effect of PENK on AGR2 expression could be seen when $scTF^-B2M^{hi}$ adenocarcinoma LuCaP 70CR (CR = castration resistant, a variant obtained from passages in castrated mice) was transfected by PENK. An increase in the expression of AGR2 was observed [75]. Increased production by LuCaP 70CR/PENK was validated by measurement of secreted AGR2 in the cell-free culture media [75]. The change in AGR2 expression indicated cancer cell differentiation induced from $AGR2^{lo}$ to $AGR2^{hi}$ by PENK in LuCaP 70CR. In contrast, AGR2 expression was down-regulated in reprogrammed LuCaP 70CR by scTF transfection to small cell carcinoma-like [60]. These results show that preventing or even reversing prostate cancer de-differentiation from luminal-like $scTF^-B2M^{hi}AGR2^{hi}$ to stem-like $scTF^+B2M^{lo}AGR2^-$ through stromal factor influence could keep anti-AGR2 therapy a viable treatment option in the disease course.

12.3 Cancer vaccine based on cancer-specificity of eAGR2

The cancer specificity of eAGR2 could allow us to develop a cancer vaccine in the future. Treated patients can be immunized by AGR2. Any emergent cells with eAGR2 expression are, by reasoning, cancerous and will be eliminated by a primed immune system. The result shown in **Figure 6** indicates no iAgr2-positive mouse organs were targeted outside non-specific background, which was non-overlapping in the study mice. Besides the bladder and lung, the intestinal tract containing $Agr2^+$ mucus-producing cells [83] also did not show labeling. Secreted AGR2 is known to function in early development where it signals cell differentiation such as that described in limb regeneration of lower vertebrates [84]. Introduction of AGR2 was reported to accelerate wound healing through recruitment of fibroblasts and migration of keratinocytes [85]. So a possibility exists that AGR2-immunized patients would experience difficulty in tissue repair after damage. For these patients, one could treat injuries with local administration of AGR2 protein.

To show the potential of an AGR2 cancer vaccine, we could immunize C57BL/6 mice with recombinant (r)AGR2 as was done in the generation of P1G4 and P3A5 (although a different mouse strain was used) [20]. After rAGR2 injection, mice are boosted at intervals and bled for ELISA testing of serum anti-AGR2 activity.

IgM-to-IgG switch is monitored. Once an adequate antibody titer is measured, syngeneic mouse bladder cancer cells MB49 [86] are implanted *vs*. into control animals without AGR2 immunization. MB49 was derived from DMBA-transformed (presumably iAgr2$^+$) bladder epithelial cells of C57BL. Whether these cancer cells express eAgr2 will be determined. If eAgr2 is not detected, we can transfect these cells with our AGR2 plasmid construct (550-bp full length cDNA cloned from prostate cancer tissue). AGR2$^-$ LNCaP cells when transfected by this plasmid produced secreted and cell surface AGR2 [81]. Note we do not need to transfect the murine Agr2 gene because the antibodies produced would recognize both human AGR2 and mouse Agr2 as shown for P3A5. We expect that the immunized mice would show no tumor growth. AGR2 vaccination will, in principle, prevent recurrence and metastasis.

12.4 AGR2 antibody in early detection

Since AGR2 expression is an early event in cancer, AGR2 antibodies could be used in early detection through imaging for example. Small foci of eAGR2$^+$ lung or pancreatic tumor can be visualized through binding of labeled chimeric P1G4. The antibody might even eradicate the detected tumors through ADCC and CDC. The iAGR2$^+$ non-involved lung epithelium would not be detected, and as would AGR2$^-$ pancreatic cells. Proposed studies will determine the minimum number of cells in a tumor mass to produce a detectable signal. Given the pg/ml detection levels of our antibodies and the high levels of AGR2 in cancer cells, this potential clinical application is promising. In addition, one could envision a reliable blood test on cancer-secreted AGR2 as a means towards cancer detection and disease monitoring.

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