

## Podoplanin

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# Podoplanin: An emerging cancer biomarker and therapeutic target

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Podoplanin (PDPN) is a transmembrane receptor glycoprotein that is upregulated on transformed cells, cancer associated fibroblasts and inflammatory macrophages that contribute to cancer progression. In particular, PDPN increases tumor cell clonal capacity, epithelial mesenchymal transition, migration, invasion, metastasis and inflammation. Antibodies, CAR-T cells, biologics and synthetic compounds that target PDPN can inhibit cancer progression and septic inflammation in preclinical models. This review describes recent advances in how PDPN may be used as a biomarker and therapeutic target for many types of cancer, including glioma, squamous cell carcinoma, mesothelioma and melanoma.

## KEYWORDS

cancer, chemotherapy, c-type lectin-like receptor 2, podoplanin

Krishnan, Rayes, Natsume and Goldberg contributed equally to this work.

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## 1 | INTRODUCTION

Podoplanin (PDPN) is a unique transmembrane glycoprotein receptor. PDPN presents a heavily glycosylated amino terminal extracellular domain of approximately 130 amino acids, followed by a single transmembrane domain of approximately 25 amino acids, and a short intracellular domain of approximately 10 amino acids. PDPN does not contain known functional domains or enzymatic activities. It utilizes other proteins, including C-type lectin-like receptor-2 (CLEC-2), heat shock protein A9 (HSPA9), CD44, galectin 8, chemokine (C-C motif) ligand 21 (CCL21), ezrin, moesin, protein kinase A (PKA) and cyclin dependent kinase 5 (CDK5), to affect cell behavior as summarized schematically in Figure 1. These ligands and binding partners interact with PDPN to control tumor cell migration, invasion and metastasis.<sup>1-4</sup>

Podoplanin expression is induced by tumor promoters including TPA, RAS and Src.<sup>5-7</sup> For example, the Src tyrosine kinase utilizes the focal adhesion adaptor protein Cas/BCAR1 to induce PDPN expression to promote tumor cell motility.<sup>5</sup> Src is a nonreceptor protein kinase that promotes nonanchored tumor cell growth and migration required for invasion and metastasis. Src is not mutated in most cancers. However, Src activity is associated with many types of human cancer, including tumors of the colon, breast, pancreas, brain and skin.<sup>8,9</sup>

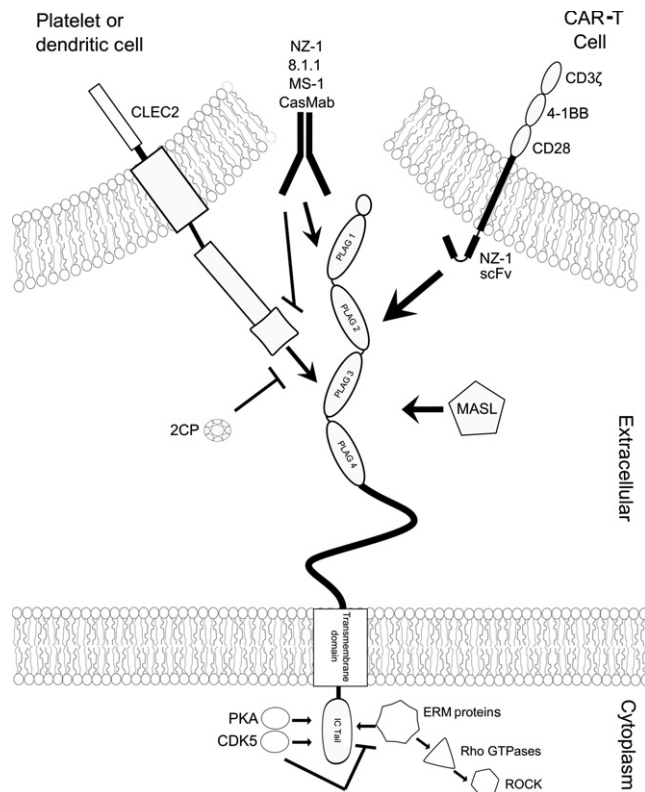
Cells transformed by a variety of chemicals, viral agents and oncogenes, including the Src tyrosine kinase, can be normalized by contact with nontransformed cells. This process, called "contact normalization" can force transformed cells to assume a normal morphology and reside in many organs, including breast, intestine and skin, for many years.<sup>10-12</sup> Comparisons between nontransformed cells, transformed cells and transformed cells undergoing contact normalization provide an extremely sensitive way to identify genes that control malignant and metastatic growth. However, Src kinase

activity alters the expression of approximately 3000 genes (approximately 10% of the transcriptome). However, fewer than 40 of these (approximately 0.1% of the transcriptome) are affected by contact normalization, with PDPN identified as a tumor promoter at the top of this list.<sup>5,12</sup>

Podoplanin expression is induced by many tumor promoters and can be found in many types of cancer.<sup>1,3,12</sup> High clonal expansion capacity is a characteristic feature of tumor initiating cells (TIC) and PDPN is a TIC marker for human squamous cell carcinoma.<sup>13</sup> Using single-cell live imaging based on the fluorescent ubiquitination-based cell cycle indicator (Fucci) system, individual PDPN expressing A431 human squamous cell carcinoma cells were shown to create large colonies more often than single A431 cells that do not express PDPN.<sup>14</sup> Although no significant differences in cell cycling were observed, cell death was significantly lower in the progenies derived from PDPN-positive single cells. RNA interference studies indicate that PDPN suppression increases cell death of single A431 cells, thus preventing them from forming larger colonies. Moreover, the frequency of large colony formation by PDPN-positive cells is decreased by treatment with a Rho-associated coiled-coil kinase (ROCK) inhibitor, whereas no difference was observed in single PDPN-negative cells.<sup>14</sup> These data, summarized in Figures 1 and 2, point to a role for PDPN in the clonal expansion capacity of TIC populations.

## 2 | PODOPLANIN AS A CANCER BIOMARKER

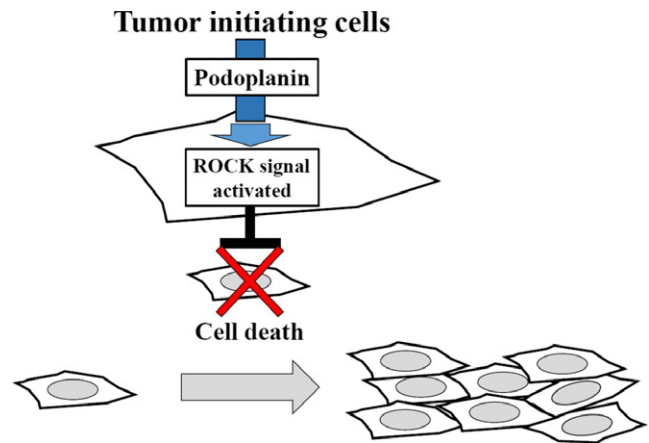
Podoplanin is expressed in several types of cancer.<sup>1,3,12</sup> Oral cancer exemplifies the utility of PDPN expression as a cancer biomarker. PDPN expression increases oral squamous cell carcinoma cell migration, which can lead to increased metastasis.<sup>15-17</sup> Accordingly, PDPN



**FIGURE 1** Podoplanin (PDPN) structure and targeting agents. PDPN contains an extracellular region, transmembrane domain, and intracellular (IC) tail. CLEC-2 interacts with PLAG domains in the extracellular region to induce inflammation and tumor progression, and this interaction can be blocked by antibodies, including 8.1.1, NZ-1, MS-1 and cancer-specific PDPN antibodies (CasMabs), as well as compounds exemplified by the small synthetic molecule 2CP. Antibodies can also target PDPN in order to inhibit transformed cell growth and motility directly, or can be incorporated into CAR-T cells. Lectins exemplified by MASL can also target PDPN to inhibit tumor progression and inflammation. protein kinase A (PKA) and cyclin dependent kinase 5 (CDK5) can phosphorylate serines on the intracellular tail to inhibit cell migration, presumably by blocking binding of ERM proteins that would otherwise lead to the activation of Rho GTPases and Rho-associated coiled-coil kinase (ROCK)

expression correlates with decreased 5-year survival rates of patients with these cancers.<sup>18</sup> Moreover, PDPN expression in precancerous oral lesions (eg oral leukoplakias) correlates with a 3-fold increase in their transformation into malignancies compared to lesions without PDPN expression.<sup>19</sup>

In addition to cancer cells, PDPN expression can be found in cancer associated fibroblasts (CAF).<sup>1,3,12</sup> For example, immunohistochemistry found PDPN expression in tumor cells from 38 out of 55 melanoma patients (69.1%). Podoplanin expression in CAF was observed in 25 of these patients (45.5%), including the 11 patients (44.0% with PDPN-positive CAF) with sentinel lymph node (SLN) metastasis. In contrast, only 4 of 30 (13.3%) patients without PDPN expression on CAF exhibited SLN metastasis. Furthermore, patients with PDPN-positive CAF experienced lower disease-free survival than those with PDPN-negative CAF ( $P = .0148$ ).<sup>20</sup>



**FIGURE 2** Podoplanin (PDPN) expression induces Rho-associated coiled-coil kinase (ROCK) activity to promote squamous cell carcinoma survival and colony expansion

In addition to histology and other standard techniques, a circulating tumor cell (CTC) chip is being developed as a blood-based marker to detect cancer. CTC are tumor cells shed from primary tumors, circulate in peripheral blood as surrogates of distant metastasis, and can be used to detect malignancies. CTC chips made of resin coated with PDPN antibodies are being developed as a microfluidic device to capture and detect CTC from metastatic cancers. For example, this technology has been used to capture and detect malignant pleural mesothelioma cells in preclinical models.

PDPN expression has been found in tumor cells as well as peritumoral basal keratinocytes which correlated with aggressive behavior in patients with extramammary Paget's disease (EMPD).<sup>21</sup> PDPN expression in peritumoral basal keratinocytes was found in 25 out of 37 patients (67.6%) with EMPD. Half (50%) of in situ EMPD cases (9 in 18) exhibited PDPN-positive keratinocytes, whereas 84.2% (16 in 19) of invasive EMPD cases demonstrated positive staining for PDPN ( $P < .05$ ). PDPN expression in peritumoral keratinocytes was also associated with tumor thickness ( $P < .005$ ). By immunohistochemical analysis, PDPN-positive peritumoral keratinocytes were found to be negative for E-cadherin, one of the major adhesion molecules of keratinocytes, which might contribute to tumor invasion into the dermis through a crack in the basal cell layer induced by downregulation of cell adhesion therein.<sup>21</sup>

Model systems are being developed to delineate how PDPN and cadherins affect each other to control tumor invasion and other events that rely on cell motility. For example, downregulation of PDPN expression by siRNA inhibits the migration of normal human epidermal keratinocytes (NHEK). This is consistent with PDPN playing a key role in this keratinocyte motility and wound healing. Interestingly, PDPN downregulation caused an increase in E-Cadherin expression, suggesting that PDPN induces NHEK migration coupled with a loss of E-cadherin. Accordingly, platelets, which express the PDPN ligand CLEC-2, inhibit keratinocyte migration. Furthermore, CLEC-2 protein itself induces E-cadherin expression, downregulates RhoA GTPase and suppresses NHEK cell migration. Taken together, these data suggest that PDPN interacts with CLEC-2 to modulate E-

cadherin expression and RhoA activity to regulate keratinocyte migration during wound healing. These results also suggest that PDPN on keratinocytes associates with CLEC-2 on platelets and delays re-epithelialization until wound bed preparation is completed during wound healing.<sup>22</sup>

### 3 | THE PODOPLANIN EXTRACELLULAR DOMAIN AS A THERAPEUTIC TARGET

Preclinical studies indicate that PDPN can be targeted to combat cancer. For example, CAR-T cells, antibodies and lectins that target PDPN can inhibit the growth and progression of glioma,<sup>23,24</sup> oral squamous cell carcinoma,<sup>17,25</sup> mesothelioma<sup>26</sup> and melanoma<sup>27</sup> in animal models. PDPN binds with CLEC-2 on platelets in the bloodstream to facilitate tumor embolism and hematogenous metastasis (Figure 1).<sup>28-34</sup> Thus, PDPN-CLEC-2 interaction offers a unique opportunity to develop anticancer strategies.<sup>35-37</sup>

Antibodies can be utilized to disrupt PDPN-CLEC-2 interaction.<sup>36,38</sup> For example, the NZ-1 antibody, its derivatives (eg NZ-8, NZ-12) and other antibodies (eg MS-1) which bind to the ectopic PLAG domain of PDPN (Figure 1) can decrease tumor load in xenograft models of glioma,<sup>36</sup> mesothelioma<sup>39</sup> and lung cancer.<sup>39,40</sup> Work with patient derived xenograft and metastasis models indicate that PDPN-CLEC-2 interaction induces platelet aggregation that promotes the extravasation step of metastasis.<sup>41</sup> This process is enhanced by growth factors and cytokines released from activated platelets during hemostasis. These factors are exemplified by TGF $\beta$ , which is released during platelet aggregation induced by PDPN on bladder squamous cell carcinoma (eg UM-UC-5) cells. Lung metastasis of these cells can be suppressed by intravenously injected administration of monoclonal antibodies specific for PDPN or TGF- $\beta$ .

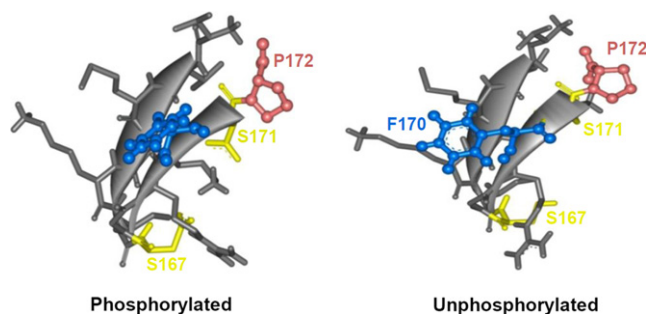
The generality of this pathway is confirmed by analysis of lung squamous cell carcinoma cells. Although PDPN expression may change over time in cell culture,<sup>34</sup> it can be found in over 60% of lung squamous cell carcinoma cells produced from fresh clinical samples. As with bladder carcinoma, xenograft models of these cells also show TGF $\beta$  released during PDPN-induced platelet aggregation, with lung metastasis suppressed by the administration of antibodies specific for PDPN. In addition to TGF $\beta$  signaling, some lung squamous cell carcinoma cells (eg PC-10) also implicate EGFR activation by platelet-derived growth factors induced by PDPN binding. This effect is suppressed by the administration of PDPN antibodies or the EGFR kinase blocker ertlotinib along with suppression of PC-10 tumor growth in vitro and in xenograft mouse models.<sup>42</sup>

In addition to antibodies, synthetic compounds are being developed to block PDPN-CLEC-2 interactions. For example, a derivative of 4-O-benzoyl-3-methoxy-beta-nitrostyrene (BMNS), compound "2CP," effectively suppresses PDPN-mediated platelet aggregation and tumor cell-induced platelet activation.<sup>33</sup> 2CP specifically binds to CLEC-2 and interacts with critical positions (Asn105, Arg107, Phe116, Arg118 and Arg157) to inhibit its binding to PDPN, as shown in Figure 1.<sup>33,43</sup> As the first defined CLEC-2 antagonist, 2CP

not only possesses anti-cancer metastatic activity but also enlarges the therapeutic efficacy of cisplatin while decreasing the risk of bleeding in experimental metastasis models.

Interactions between PDPN and CLEC-2 can also be blocked to modulate the inflammatory response in sepsis, which is often associated with cancer progression and treatments. Indeed, sepsis is a life-threatening, severe systemic inflammatory response associated with multiple organ failure and death, which affects over 19 million patients annually.<sup>44,45</sup> Thrombocytopenia is common in sepsis and severe thrombocytopenia is associated with poor outcome in septic patients and mice.<sup>46-48</sup> Platelets are now recognized as critical immunomodulators affecting immune cell recruitment, releasing cytokines and chemokines and trapping bacteria.<sup>49</sup> Platelet depletion or inhibition of platelet activation results in a decrease in survival from sepsis.<sup>49,50</sup> Moreover, platelets maintain vascular integrity at the site of inflammation through the PDPN and collagen/fibrin receptors, CLEC-2 and glycoprotein VI (GPVI), respectively.<sup>51-53</sup> In sepsis, platelet interaction with inflammatory macrophages dampens macrophages pro-inflammatory phenotype and decrease the secretion of TNF- $\alpha$ .<sup>50</sup> Recent studies indicate that platelet CLEC-2 interaction with PDPN on inflammatory macrophages regulates the immune response in a mouse model of sepsis, cecal ligation and puncture (CLP). Platelet deletion of CLEC-2 or PDPN-deficient hematopoietic cells increased the clinical severity of sepsis associated with enhanced systemic inflammation and accelerated organ injury. Deletion of CLEC-2 from platelets or PDPN from macrophages potentiates the cytokine storm and reduces PDPN expressing inflammatory macrophage migration to the infected peritoneum. In addition, pharmacological inhibition of the CLEC-2-PDPN axis inhibits immune cells infiltrate at the site of infection and regulates their inflammatory phenotype.<sup>54</sup> These observations identify PDPN as a novel anti-inflammatory target regulating immune cell recruitment and activation in sepsis.

In addition to antibodies and synthetic molecules, lectins may be used to target PDPN on transformed cells. For example, *Maackia*



**FIGURE 3** Predicted structural conformation of the intracellular domain of mouse podoplanin (PDPN) in the phosphorylated and unphosphorylated states. The intracellular domain of PDPN contains serine residues (yellow) that can be modified to affect cell motility. Least energy structural conformation calculated by PEP-FOLD predicts an alteration in the orientation of an intracellular phenylalanine residue (blue) that correlates with decreased cell migration



*Amurensis* seed lectin (MASL) binds to PDPN on melanoma and oral squamous cell carcinoma cells to inhibit their motility and growth in vitro and in syngeneic and xenograft mouse models (Figure 1). Interestingly, both MASL and NZ-1 antibody decrease tumor cell migration at nanomolar concentrations, apparently by inhibiting Cdc42 GTPase activity, and kill cells by nonapoptotic caspase independent necrosis at higher micromolar concentrations.<sup>17,27</sup>

#### 4 | TARGETING THE INTRACELLULAR PODOPLANIN DOMAIN

The intracellular domain of PDPN contains only 10 amino acids, including basic amino acids such as lysines and arginines. These basic amino acids act as binding sites for the ezrin family proteins. Upon binding to the intracellular domain of PDPN, the ezrin family proteins modulate Rho GTPases and reorganize the actin cytoskeleton to promote cell migration, as shown in Figure 1.<sup>55</sup>

In addition to basic amino acids, the intracellular domain of PDPN also contains 2 conserved serine residues, which were long considered to be putative phosphorylation sites.<sup>15,56,57</sup> The functional relevance of these serine residues was elucidated by mutagenesis and cell motility experiments. Interestingly, phosphorylation of serines inhibits PDPN-mediated cell migration. Furthermore, both serines need to be phosphorylated to inhibit cell migration.<sup>4,58</sup> Phosphorylation can modify the structural conformation of amino acids in the PDPN intracellular domain, as shown in Figure 3.

The kinases that can phosphorylate PDPN cytoplasmic serine residues were identified as protein kinase A (PKA) and cyclin-dependent kinase 5 (CDK5), as shown in Figure 1. While PKA can phosphorylate either of the 2 serines (S167 or S171 in mouse PDPN), CDK5 preferably phosphorylates the C-terminal serine (S171 in mouse PDPN).<sup>4</sup> These data suggest a scenario in which PKA and CDK5 work together to phosphorylate the intracellular serines of PDPN in order to inhibit cell motility. Reagents that can induce PDPN phosphorylation may be used to inhibit tumor motility. For

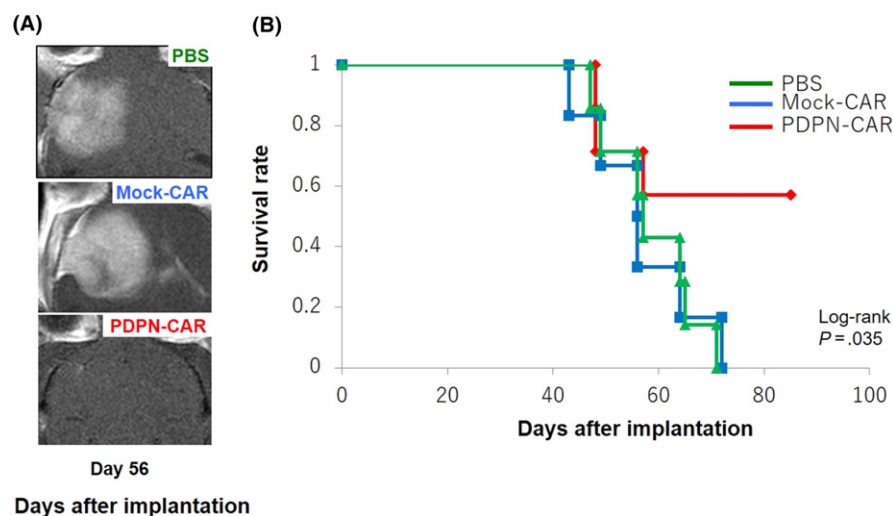
example, 8-br-cAMP, disulfiram and CARP-1 functional mimetics have been shown to induce PDPN phosphorylation and inhibit PDPN-mediated cell migration.<sup>4,59,60</sup> Thus, PDPN may be targeted both on its intracellular domain as well as its extracellular domain to inhibit cell migration.

#### 5 | PODOPLANIN CAR-T CELLS

CAR-T cells targeting PDPN are being developed to treat cancer. This is exemplified by recent work focused on glioblastoma. Glioblastoma (GBM) is the most common and lethal primary malignant brain tumor in adults, with a 5-year overall survival rate of less than 10%.<sup>61</sup>

Chimeric antigen receptors (CAR) consist of an extracellular domain derived from a single-chain variable fragment (scFv) taken from a tumor antigen-specific monoclonal antibody (mAb), a transmembrane domain, and a cytoplasmic signaling domain CD3 $\zeta$  chain (CD3 $\zeta$ ) derived from the T-cell receptor complex.<sup>62</sup> CAR-transduced T cells can recognize predefined tumor surface antigens independent of major histocompatibility complex (MHC) restriction, which is often downregulated in gliomas.<sup>63</sup> Third generation CAR, that include 2 costimulatory domains such as CD28 and 4-1BB (CD137), have been described and are highly likely to lyse tumor cells.<sup>64</sup>

Several CAR have been generated against antigens expressed in GBM, including epidermal growth factor receptor variant III (EGFRvIII), human epidermal growth factor receptor 2 (HER2), interleukin-13 receptor alpha 2 (IL13R $\alpha$ 2), and, as described here, PDPN.<sup>24</sup> In particular, a lentiviral vector has been constructed with the EF1 $\alpha$  promoter followed by the leader sequence, NZ-1 PDPN antibody-based scFv, CD28, 4-1BB and CD3 $\zeta$ . The lentiviral vector was used to infect human T cells. A calcein-based nonradioisotope cytotoxic assay indicated that PDPN-positive LN319 cells and U87MG glioma cells were lysed by these NZ-1-CAR-T cells in an effector/target (E/T) ratio-dependent manner.<sup>24</sup> In contrast, specific lysis was not observed against PDPN-knockout (KO)-glioma cells.



**FIGURE 4** CAR-T cells targeting podoplanin (PDPN) inhibit glioblastoma progression in orthotopic xenograft mice. (A) Post-treatment MRI. (B) 60% of the mice treated with NZ-1 CAR-T were cured

In addition, NZ-1-CAR-T cells co-cultured with PDPN expressing glioma cells released significantly more IFN $\gamma$  than mock-transduced T cells.<sup>24</sup>

An intracranial glioma xenograft model was used to examine the distribution and anti-tumor effect of NZ-1-CAR-T cells.<sup>24</sup> To this end, glioma cells were stereotactically implanted into an immunodeficient mouse brain. Seven days after tumor implantation, NZ-1-CAR-T cells or mock-transduced T cells were infused intravenously via the tail vein. The non-treated mice were infused with PBS alone, and intracranial tumor growth was evaluated by 3T-MRI. In approximately 60% of the mice treated with NZ-1-CAR-T cells, tumors grew markedly slower and the mice survived significantly longer than control groups, as shown in Figure 4. Taken together, these data indicate that functionally active NZ-1-CAR-T cells recognize PDPN to inhibit glioma cell growth and tumor progression.

## 6 | CONCLUSIONS AND FUTURE PERSPECTIVES

Cancer is extremely complex and heterogeneous, in which the underlying factors are often poorly understood at the level of individual patients. PDPN is expressed by many types of tumor cells and CAF. Moreover, high levels of PDPN expression is associated with reduced survival and cancer aggression. PDPN has clear potential as a cancer biomarker and therapeutic target. These therapies include a variety of compounds, biologics, antisera and CAR-T cells as summarized in Figure 1.

One concern with PDPN CAR-T therapy arises from nonspecific lysis of normal cells that express PDPN, including lymphatic endothelium and type I lung alveolar cells. Cancer-specific monoclonal antibodies (CasMabs) have been generated to address this concern. These PDPN CasMabs react with PDPN expressed by cancer cells, but not normal cells.<sup>65</sup> These should be extremely useful reagents to produce very specific CAR-T therapies that target PDPN to combat glioma and other cancers.

As with most other anticancer therapies, it is important to understand which patients are likely to benefit from anti-PDPN treatments, such that each patients' therapeutic program can be tailored to their specific disease. Histopathological examination, often supported by clinical imaging (MRI or CT) and findings during surgery can be used to classify PDPN in patient tumors. However, direct, functional assessment of drug responses on primary patient-derived tumor cells gives the most accurate information on whether the patient will respond to the tested drugs. For example, zebrafish tumor xenograft platforms allow human tumor samples to be grafted into zebrafish embryos, where their growth as primary tumors and their dissemination to distal regions can be determined in the presence or absence of drugs.<sup>66-68</sup> This platform has been used to demonstrate efficacy of the anti-PDPN compounds, including MASL on oral squamous cell carcinoma and melanoma xenografts.<sup>17,27</sup> This approach can be used to gather

critical information that can be reported back to oncologists in charge of treatment planning in less than 5 days after surgery.

## CONFLICT OF INTEREST

Gary Goldberg has intellectual property and ownership in Sentrimed, which is developing agents that target PDPN to treat disease, including cancer, and received funding from the New Jersey Health Foundation to develop methods to target PDPN to treat cancer. The other authors have no conflicts of interest to declare.

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