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REVIEW

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Current drug design to target the Semaphorin/Neuropilin/Plexin complexes

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ABSTRACT

The Semaphorin/Neuropilin/Plexin (SNP) complexes control a wide range of biological processes. Consistently, activity deregulation of these complexes is associated with many diseases. The increasing knowledge on SNP had in turn validated these molecular complexes as novel therapeutic targets. Targeting SNP activities by small molecules, antibodies and peptides or by soluble semaphorins have been proposed as new therapeutic approach. This review is focusing on the latest demonstration of this potential and discusses some of the key questions that need to be addressed before translating SNP targeting into clinically relevant approaches.

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Introduction

The first semaphorin (at that time called collapsin) has been discovered in 1993 by the group of JA Raper.[1] This secreted protein turned out to be a strong inhibitor of axon growth by inducing rapid depolymerization of the actin cytoskeleton. This property consequently was shown to transiently destabilize the axonal growth cone thereby impeding proper axonal guidance. From this discovery, a huge amount of data has been generated over the last 2 decades that describe the now called semaphorin family and its pleiotropic functions. Almost all organs and tissues express several semaphorins from early development up to the adult which occurs in both healthy and damaged tissues. The many properties of semaphorins[2] can explain the diversity and multitude of functions they have on cell death, proliferation, migration and differentiation. Consequently, dysregulated semaphorin expression or signaling is associated with a variety of diseases in the central nervous system (CNS), the cardio-vascular system (CV), the immune system (IS) and cancer.

However, the main challenge when studying the semaphorin functions is their extraordinary versatility inherent to their many signaling pathways. Semaphorins can exhibit promoting or inhibiting effects depending on the receptor complex.[3-6] Indeed, the class 3 semaphorins (secreted molecules) bind to Neuropilins that in turn associate with co-receptors to trigger cellular signaling. Neuropilin-1 (NRP1) which was initially identified as a cell surface receptor in the nervous system,[7] is the primary class 3 semaphorin binding partner[8] with the exception of Sema3E which directly binds Plexin D1.[9] NRP1 lacks direct signaling activities and therefore needs to associate with co-receptors to transduce the semaphorin signal. It has been largely described that Plexins are the main co-receptors of NRP1 to transduce class 3 semaphorin signaling while transmembrane semaphorins can directly interact with Plexins .[10,11] Different combinations of Plexins and NRP1 can generate a variety of complexes that modulate semaphorin signaling.[12] Moreover, additional coreceptors such as the cell adhesion molecule L1 for Sema3A[13] or CD72 for Sema4D[14] have also been described. In addition, NRP1 and its homolog Neuropilin-2 (NRP2) do not only bind semaphorins but also bind VEGF and several other molecules.[15] These interactions are evidently important to control normal and tumor associated angiogenesis.[16] Moreover, NRPs interact with many other growth factors such as FGF, PDGF or TGF-β1 and their cognate tyrosine kinase receptors (RTKs).[17] NRPs are therefore entering the complex hub of RTKs and their intricate network of signaling pathways. This already large signaling versatility was even further increased when NRPs were identified as partners of integrin signaling[18] that could regulate fibronectin fibril assembly.[19] Hence, besides transmembrane

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semaphorins which do not seem to require NRPs for signaling[10] most of the secreted semaphorins compete with several other ligands (directly or indirectly) involving NRPS to establish their bona fide signaling pathways.

The obvious diversity of semaphorin/Neuropilin/Plexin (SNP) complexes and their involvement in diseases raises the frequently asked question whether they are good therapeutic targets.[20] Indeed, several approaches have been tested to regulate semaphorin signaling either by inhibitors or by using the soluble molecule itself. An excellent review by Mishra and colleagues[21] is providing detailed information about semaphorins and NRPs. Here, this review will summarize *in vivo* studies that were undertaken to evaluate SNP complexes as potential targets in curing various pathologies.

Using soluble semaphorins as therapeutic agents

Numerous studies have shown that semaphorins can act as inhibitors of cell migration, cell proliferation or even inducers of apoptosis. Their pro-apoptotic role is particularly interesting in the cancer context. Another good reason to consider regulation of semaphorins in tumor development is the clear anti-angiogenic activity of class 3 semaphorins that has been proven in several models.[22-26] Thus, it is tempting to use the natural properties of semaphorins to trigger inhibition of tumor cell growth and/or tumor angiogenesis by synthetic versions of semaphorins. Indeed, intraocular injection of Sema3E displays an anti-angiogenic activity on developing normal vessels through binding to Plexin D1.[27] While affecting also tumor associated vessels, a negative impact on normal vessels may represent a major risk of bleeding as side effect in some tissues. However, intravitreal administration of the Sema3E protein selectively suppressed extraretinal vascular outgrowth without affecting the regeneration of the retinal vasculature in a model of ischemic retinopathy (Fig. 1).[28] The use of semaphorins as therapeutic agents to treat abnormal vessel development is again strengthened by the demonstration that Sema3C inhibits pathological angiogenesis in a murine oxygen-induced retinopathy model.[29] In these studies, recombinant proteins were locally administrated. It would be interesting to know whether systemic administrations could provide similar beneficial effects. Consistently, a stabilized form of Sema3C (a furin cleavage-resistant Sema3C) was able to inhibit tumor angiogenesis as well as tumor lymphangiogenesis and tumor.[30] The continuing effort devoted to analyze the therapeutic potential of additional semaphorins is also fruitful in other diseases, as the transmembrane Sema4B was shown to inhibit non-small cell lung cancer growth *in vivo* when overexpressed in the cancer cells.[31] Because gene delivery-based therapies are still under debate and somehow still difficult to achieve, it remains to be shown whether administration of a soluble form of the protein would be sufficient to recapitulate the effects of ectopically expressed Sema4B. The same comment applies to glioblastoma, where Sema3D or Sema3E when ectopically expressed by the tumor cells (using lentivirus-based strategies) reduced tumor growth.[32] It would be interesting to see whether local or systemic administration of recombinant Sema3D and Sema3E molecules are able to reach the tumor and block glioblastoma growth, where, despite a high tumor blood vessels leakiness, crossing the blood brain barrier may represent an issue (Fig. 1). Alternatively, lentivirus delivery *in vivo* (by direct intracerebral injection) into established tumors should be tested because a similar strategy applying AAV-mediated delivery and expression of Sema6A in the cortex enhanced post-ischemic recovery of animals (Fig. 1).[33] This study showed that at least membrane bound semaphorins can be produced at the right place, in the right cells and at the right concentration to exert therapeutic effects. These expression properties will certainly be more difficult to achieve with secreted semaphorins because autocrine effects or gradient mediated-effects may generate cell type specific and opposing results. Sema3A is an example to illustrate this complexity as it can stimulate glioma cell dispersion when being overexpressed by the tumor cells[34] while it inhibits breast tumor growth when delivered systemically.[35] A similar tumor growth inhibitory effect has also been reported for head and neck squamous cell carcinoma upon intratumoral injections of Sema3A-encoding adenoviruses (Fig. 1).[36] Another interesting option comes from the description of the Sema3C-dependent promotion of dopaminergic axons in view of cell therapy for Parkinson disease.[37] In this *in vitro* study, Sema3C was incorporated in a hydrogel (PuraMatrix) to ensure stable long-term release and trigger enhanced axon outgrowth, thus arguing for the use of use such biocompatible hydrogels to deliver semaphorins *in vivo* as e.g. as inhibitors to block tumor growth (Fig. 1).

Thus, semaphorins can be considered as therapeutic agents, yet the delivery mode needs to be solved. Moreover, the biodistribution profiles should be determined as a function of the administration mode. A special attention should also be given to the semaphorins that are not involved in the maintenance and integrity of adult tissues as a recent study showed that an excess of Sema3A causes severe diabetic nephropathy[38] and neuronal toxicity.[39] Future development is also needed to increase stability, target binding, and preferential delivery to only abnormal tissues. In this context it is interesting to note the study from the Tamagnone laboratory

who investigated local delivery of an uncleavable Sema3E (by using an Alzet osmotic mini-pump) and observed inhibition of tumor angiogenesis in the pancreatic Rip1-Tag2 model (Fig. 1).[40]

Using small molecules as inhibitors of semaphorin signaling

Inhibitory small molecules represent a very classical approach. In case of SNP complexes, very few studies have however tried or succeeded by using small interfering molecules. Xanthofulvin and Vinaxanthone are specific natural inhibitors of Sema3A (refs.[41,42,43]) and showed strong regenerative properties in a model of spinal cord lesion.[44] Recent work identified a synthesis procedure helping to characterize the structures of the compounds.[45,46] Such a better characterization of the compounds should now lead to a better understanding of the mode of action, a prerequisite to further develop these compounds as therapeutics. Another mode of semaphorin inhibition is provided by the so called ligand-caging system that predominantly uses soluble forms of SNPs such as NRP1 or NRP2 (refs. [47, 48]). As an example, expression of the Sema3E ligand trap derived from Plexin D1 (SD1, containing the Sema domain of Plexin D1) by the tumor cells reduced tumor growth.[49] A multi-step in silico / *in vitro* screening procedure recently led to the identification of a non-peptidic VEGF-A165/NRP protein-protein interaction antagonist.[50] Based on this molecule, several other antagonists were recently designed.[51] While originally designed to selectively block VEGF binding, it would be interesting to determine whether these compounds potentially also affect semaphorin binding and signaling. Any compound antagonizing both semaphorins and VEGF ligand binding would certainly be interesting in the context of cancer since both pathways are strong promoters of cancer.

Using function blocking antibodies to inhibit semaphorin signaling

Currently, except small molecules monoclonal antibodies represent the most important targeted therapeutics. Several antibodies have been generated to block semaphorins or their receptors. Using a neutralizing monoclonal antibody targeting Sema3A, the group of Y. Goshima was able to ameliorate lipopolysaccharide-induced sepsis in mice.[52] Considering the numerous conditions involving abnormal Sema3A signaling, this antibody may represent a very interesting therapeutic tool with a large spectrum of indications. A neutralizing antibody had also been generated against the transmembrane Sema4D and was shown to markedly prevent bone loss in a model of postmenopausal osteoporosis when administrated every 3 days for 3 weeks.[53] Additional Sema4D blocking antibodies have been developed and characterized in various disease conditions including cancer,[54,55] Huntington disease,[56] experimental autoimmune encephalomyelitis[57] and rheumatoid arthritis.[58] The Sema4D blocking VX15/2503 antibody is currently in clinical studies (conducted by Vaccinex Inc. in Huntington disease and non-small cell lung cancer) after successful preclinical characterization and thanks to a good tolerance profile in human patients (Fig. 2).[59] Few years ago, the structural analysis of the extracellular domain of NRPs described 2 independent binding sites for semaphorins and VEGF,[60] thereby providing an interesting rational for the design of function blocking antibodies. Until today, NRP1 is the most advanced target for antibody development. The MNRP1685-A antibody that targets the VEGF-binding domain has been evaluated in a promising phase 1 study[61] followed by a phase 1B study showing unexpected side effect (high proteinuria) in combination with Bevacizumab (anti-VEGF) with or without paclitaxel (Fig. 2).[62] An interesting study recently showed that the Fab' fragment of a mouse NRP1 antibody can be used to functionalize cytotoxic drug-containing liposomes. This turned out to be an effective method to concentrate the drug at the tumor site. The confirmation of such results with a humanized antibody could represent an interesting therapeutic potential. In addition, the description of an anti-NRP2 antibody[63] with strong anti-metastatic properties is highly interesting (Fig. 2), yet this approach has not been further pursued for clinical translation. Moreover, despite development of a new anti-NRP2 antibody that targets the b1b2 domain[64] functional *in vivo* assays are missing. Function blocking antibodies were also raised against Plexins. Based on its promoting role in acute inflammation,[65] Plexin C1 may be a good therapeutic target. Indeed, a function blocking antibody against Plexin-C1 reduced hepatic ischemia-reperfusion injury by a mechanism that blocked transmigration of neutrophils.[66] One major difficulty when targeting the SNP complexes remains the redundancy of semaphorins and the multiplicity of the receptors which are moreover shared with several other ligands. This implies to develop smart compounds overcoming this bottleneck to produce selective but very potent molecules that bypass the competition of ligands and block the multiple receptors involved in the signaling.

Using antagonist peptides to target or inhibit SNP complexes

The development of therapeutic peptides is growing incredibly fast in the pharmaceutical race.[67] If

therapeutic peptides such as insulin have undoubtedly shown their efficacy and safety,[68] the classical challenges of peptide and protein immunogenicity[69] or delivery[70] slows down the emergence of new drugs in comparison to the development of small molecule inhibitors (Fig. 2). Peptides mimicking ligands can be used to antagonize receptor binding, or can be used to mirror the biological activity of the ligand. Consistently a wide range of agonistic or antagonistic peptides can be developed by partially or totally using the original sequences of the respective molecules. Chemical modifications can also be applied to stabilize the structure and/or the stability of therapeutic peptides. In case of SNP complexes, one of the first peptide-based strategy has been proposed by Doherty and colleagues who characterized a peptide targeting the extracellular MAM domain of NRP1 (Fig. 2).[71] The recent publication of the crystal structure of the MAM domain may provide interesting information to optimize docking of peptides in this crucial domain.[72]

Another interesting approach designed a peptide with a motif recognizing the VEGF binding domain of Neuropilin-1.[73] The latest development of this approach led to the design of multifunctional nanoplatforms exhibiting strong potential for photodynamic therapy of brain tumors.[74] Cationic peptides and peptidomimetics have also been developed to antagonize Sema3A functions presumably by interfering with Sema binding to glycoaminoglycans.[75] A peptide recognizing the VEGF binding domain of NRP1 showed interesting inhibitory properties in experimental rheumatoid arthritis (Fig. 2).[76]

An unexpected alternative approach emerged from studies of the so far largely ignored transmembrane domain (TMD) of Neuropilin-1.[77] Indeed, the transmembrane domain of bitopic receptors is usually considered as a membrane anchoring element without further functions. However, the TMDs play a crucial role in receptor dimerization and together with juxta membrane domains they are able to control and modulate

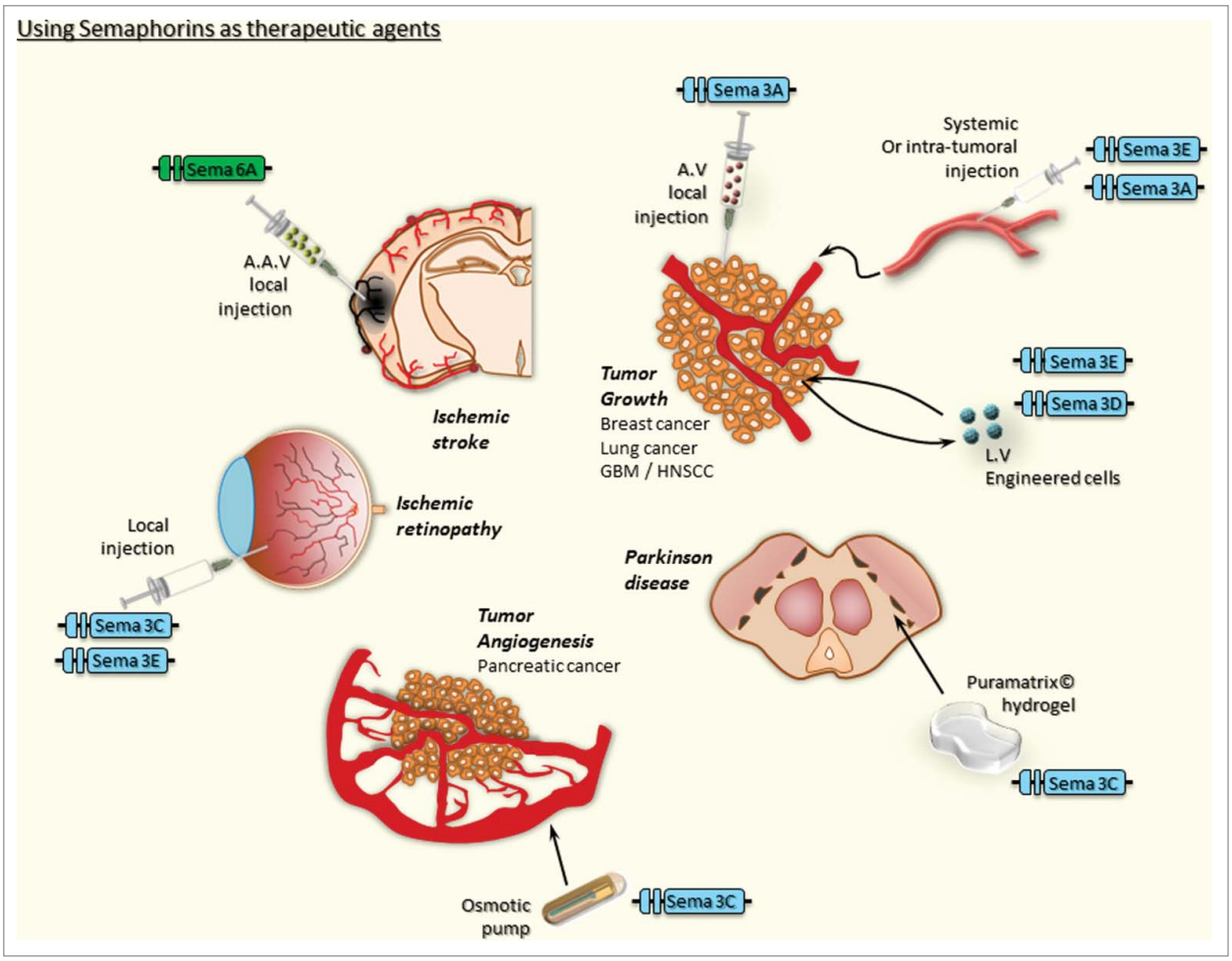

Figure 1. Using semaphorins as therapeutic agents. This cartoon is illustrating the major pathological conditions in which semaphorins have been shown to produce a potential therapeutic effect. The delivery mode is mentioned for each experimental *in vivo* models. AV, Adenovirus; AAV, Adeno-associated virus; LV, Lentivirus.

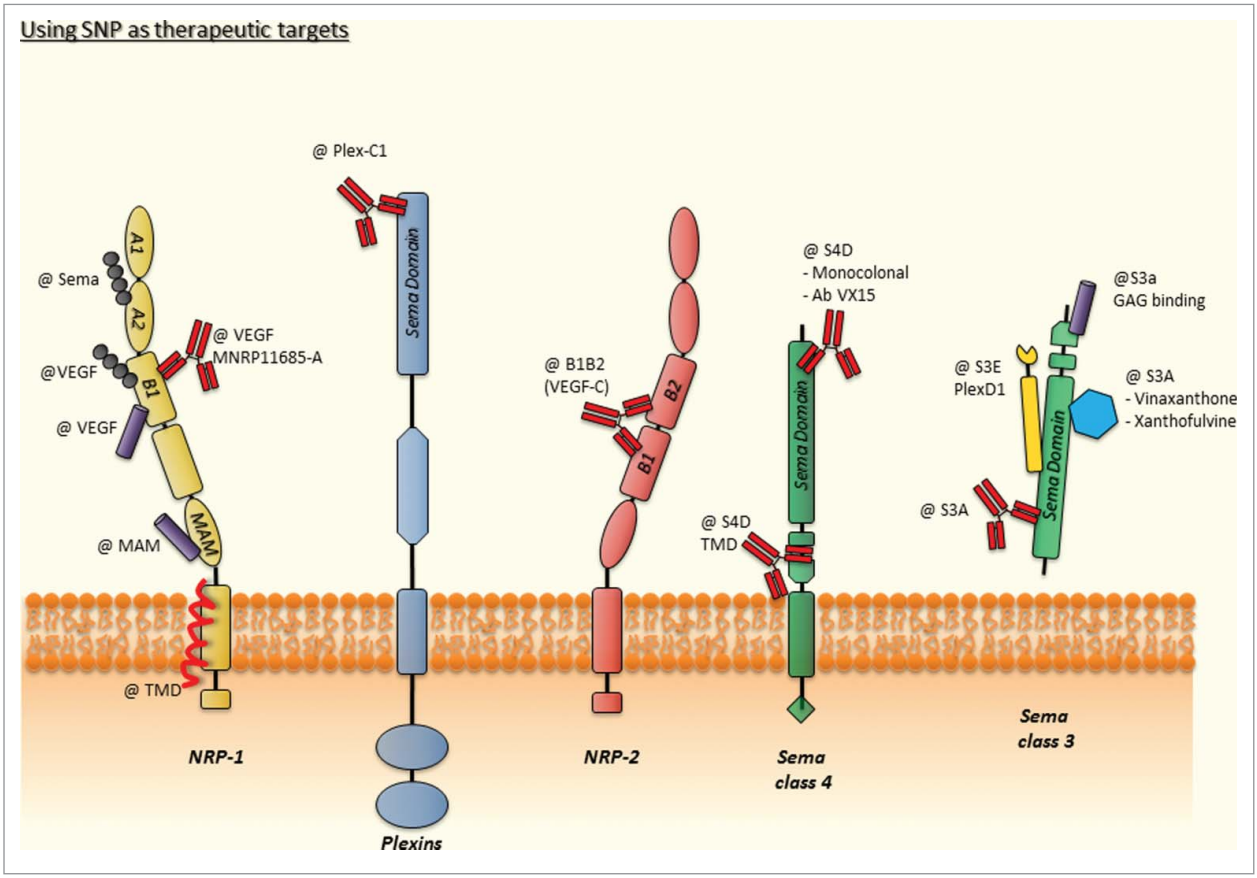

Figure 2. Using SNP as therapeutic targets. This schematic representation of SNP is integrating the different types of drugs targeting the extracellular or membrane domains. TMD, Trans Membrane Domain; Ab, Antibody.

extracellular domain ligand-binding capacity or structure.[78,79] Several mutations in TMD are associated with severe human diseases in cancer.[80] The existence of dimerizing motives such as the GAS motives[81] is known to be the source of selective TMD interactions either for homo- or hetero-dimerization. The TMDs of Neuropilins and Plexins are indeed now well characterized and several studies have shown the specificity of interactions.[82] The systematic analysis of TMD / TMD interactions is progressively contributing to the demonstration of a TMD interaction code defining very precise and specific rules of receptor dimerization and heterodimerization. Consistently, peptides targeting the TMD of NRP1 act as receptor dimerization inhibitor leading to the blockade of related biological functions.[77] This property turned out to be an efficient strategy to fight against glioblastoma growth *in vivo* with the corresponding Membrane Targeting Peptide MTP-NRP1 (Fig. 2).[83] The strength of such therapeutic peptides is their relative independence toward ligand binding and their intrinsic capability to selectively and specifically interfere with several receptors. Strikingly, such TMD targeting peptides can exhibit biological effects in preclinical *in vivo* models with a very low dose of 1 μg/kg (or 10 μg/kg for peptides targeting Neu/ ErbB2 receptor),[84] doses delivered every 3 days. Such treatments are well tolerated even when administrated for several weeks.[85] Another exciting feature is the recent demonstration of the anti-metastatic effect of MTP-NRP1 in a triple negative preclinical breast cancer model.[85] In this study, it was shown that MTP-NRP1 could exert a protective effect by reducing metastases growth if administrated before grafting of metastasizing cells. This result suggests the possibility to design preventive treatments attacking the metastasis process upon detection of a primary tumor before metastasis occurs.

Conclusion

The SNP complexes clearly define an exquisite molecular target for drug design. The way to produce drugs tackling these molecules however is not solved yet because of the functional versatility of the SNP complexes. Promoting the natural inhibitory effects of semaphorins in one condition may have deleterious effects in another organ while blocking the semaphorin receptors may counteract positive effects of semaphorins in certain disease contexts. The existence of gradients of soluble semaphorins

and the highly diverse and dynamic composition of the receptor complexes are creating additional complexity. A detailed analysis of the biodistribution and safety profiles for SNP targeting drugs is urgently required. The next step will be to study the combination of SNP targeting drugs together with other anti-cancer drugs to address the question of additive, compensatory or resistance mechanisms. Hence, the dual targeting and inhibitory properties of SNP drugs could be better explored by designing smart compounds such as nanoparticles to ensure carrier-mediated specific delivery to damaged tissues while normal tissues are spared. If the last decade was asking the question whether SNP complexes were potential therapeutic targets, the next decade will be the one to clarify what are the best drug design strategies and application modes.

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