LGR4: Not Just for Wnt Anymore?

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Leucine-rich repeat-containing G protein–coupled receptor 4 (LGR4) is best known for its role in regulating the ability of cells to respond to Wnt ligands. In this well-known role, LGR4 serves as a receptor for R-spondins and forms a complex with the ubiquitin E3 ligases ring finger protein 43 (RNF43) and zinc and ring finger 3 (ZNRF3). RNF43 and ZNRF3 ubiquitinate Frizzleds (FZD), which are a family of ten WNT receptors. This ubiquitination decreases FZD receptor levels on the cell surface, reducing Wnt ligands' ability to activate signaling. While there were some previous indications of Wnt-independent functions of LGR4, this WNT-centric view has remained predominant. In this issue of *Cancer Research*, Yue and colleagues report that LGR4 also functions to regulate signaling through the EGF receptor. This work was stimulated by observing that while high levels of LGR4 expression in breast tumors correlated with poor patient outcomes, LGR4 levels did not correlate with a well-established Wnt-associated gene signature in these same patients. In contrast, high levels of Lgr4 expression strongly correlated with EGFR signaling. Reducing Lgr4 expression also inhibited signaling through the EGFR, potentially via regulation of the Casitas B-lineage lymphoma ubiquitin E3 ligase. Consistent with this model, LGR4 could be coimmunoprecipitated with a complex that contained EGFR and was capable of inhibiting EGFR ubiquitination. The implications of this work and how it challenges our understanding of the contributions of Wnt signaling and EGFR signaling in cancer are discussed as our several interesting future directions.

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Leucine-rich repeat-containing G protein–coupled receptor (LGR4) is a member of the Type B subfamily of rhodopsin G protein-coupled receptors (GPCR; ref. 1). Members of this subfamily, which also includes LGR5 and LGR6, contain a large N-terminal extracellular domain with 17 tandem copies of a leucine-rich repeat. LGR4 was identified in 1998, but its ligand was not identified until 2011, when all four members of the R-spondin family (RSPO1–4) were shown to bind to LGR4, LGR5, and LGR6 (LGR4/5/6).

Our current understanding of the LGR4/5/6 family focuses on their interactions with the R-spondin ligands and concurrent interaction with E3 ubiquitin ligases (1). Together, these events lead to the downregulation of Wnt signaling. Wnt ligands engage a receptor complex that includes a member of the Frizzled (FZD) family of seven transmembrane receptors and a coreceptor to activate downstream signaling (2). The levels of FZD proteins are regulated by ubiquitination, which targets them for proteolytic degradation. FZD ubiquitination is regulated by the activity of two single-pass transmembrane E3 ubiquitin ligases, ring finger 43 (RNF43) and zinc and ring finger 3 (Zrnf3; ref. 1). RNF43 and ZNRF3 directly ubiquitinate FZD proteins on cytoplasmic lysine residues to target them for degradation. R-spondins bind both to RNF43 or ZNRF3 and either LGR4, LGR5, or LGR6, resulting in the internalization of that complex. Thus, R-spondins reduce RNF43/ZNRF3 levels in an LGR4/5/6–dependent manner. This increases FZD levels, thereby sensitizing cells to Wnt-mediated signaling.

During the last decade, the importance of understanding the molecular mechanisms regulated by these complexes has grown. For example, almost all cocktails of growth factors that have been developed for the culture and maintenance of tissue organoids include R-spondins (3). Thus, a better understanding of the functions of R-spondins and LGR4/5/6 proteins will have implications for interpreting data using such systems. Furthermore, mutations in components of this complex are associated with a variety of human tumors (2). These include translocations that lead to increased or ectopic R-spondin expression and inactivating mutations in RNF43 or ZNRF3. The effects of these mutations have been interpreted almost solely in the context of their role in regulating Wnt signaling. Some previous work demonstrated that LGR4 and its interaction partners had broader functions beyond regulating the level Wnt receptors to control signaling (4, 5). However, the prevailing thought has remained that LGR4/5/6 and their interacting partners impact cellular signaling and phenotypic outcomes primarily via their effects on the Wnt pathway.

In this issue of *Cancer Research*, Yue and colleagues present additional information that directly challenges this Wnt-centric view (6). The authors set out to examine the role of LGR4 in breast cancer growth and metastasis. This was partially stimulated by the puzzling observation that although high LGR4 expression was strongly associated with poor metastasis-free survival in triple-negative breast cancer patients, a well-validated Wnt/β-catenin signature was not. They then demonstrated that either chemical inhibition of Wnt with porcupine inhibitors, which inhibit Wnt production, or deletion of Wntless, a Wnt-specific chaperone protein necessary for secretion of all Wnt ligands (7), inhibited Wnt signaling but did not impair the ability of LGR4 to promote migration and invasion. In addition, point mutant versions of LGR4 that lack the ability to potentiate Wnt signaling retained the capacity to promote cell motility *in vitro* and metastasis *in vivo*.

The authors then performed shRNA knockdowns of LGR4 and evaluated proteins that were altered in this setting. Reductions in LGR4 expression resulted in changes in levels and activation status of the epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), and SRC-family kinases. Further investigation showed that EGFR and HER2 levels increased after LGR4 overexpression, consistent with previous reports that EGFR expression was decreased in the tissues of *LGR4* knockout mice. Furthermore, knockdown of EGFR, or inhibition of EGFR with erlotinib, attenuated LGR4-

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induced cell migration and invasion. Collectively, these data present a convincing case that EGFR activity is required for LGR4 to promote breast cancer progression.

In a final set of experiments, the authors set out to identify a mechanism by which LGR4 could regulate EGFR. Knowing that EGFR levels are regulated by the activity of the E3 ubiquitin ligase, c-CBL (CBL; ref. 8), they examined how the loss of LGR4 would affect EGFR-CBL interactions. They found that loss of LGR4 enhanced CBL binding to EGFR, suggesting that LGR4 functions impair the CBL–EGFR interaction. They also demonstrated that LGR4 could be detected in immunoprecipitates with EGFR antibodies, leading them to suggest that LGR4 physically interacts with EGFR signaling complexes, subsequently preventing CBL-mediated ubiquitination of EGFR. The resulting increase in EGFR signaling potentiates breast cancer metastasis.

This strong linkage of LGR4 to EGFR and HER2 has high translational relevance (9). Mutations or alterations in EGFR are associated with almost 30% of all epithelial cancers, while HER2 amplifications define a whole class of breast cancer. Interestingly, HER2 does not interact with CBL as strongly as EGFR, and thus EGFR/HER2 heterodimers are more resistant to CBL degradation. In addition to the observed correlations between EGFR and LGR4 in patient samples, it will also be interesting to evaluate the association of increased LGR4 expression with HER2 alterations. This LGR4 connection could provide a potential explanation for why HER2 is relatively more resistant to CBL-initiated degradation than EGFR.

An obvious possibility that has emerged from this manuscript is that therapeutically targeting LGR4 could be a strategy that would allow for reductions in both EGFR signaling and Wnt-mediated activation of downstream pathways. It will be of interest to see if the efficacy of such strategies would be similar to simultaneously inhibiting those pathways via independent mechanisms.

Beyond the potential therapeutic opportunities, this manuscript may significantly change how we view the functions of LGR4/5/6 and the R-spondins. For example, does the addition of R-spondins to organoid and cell culture media act via potentiating both EGFR and Wnt-mediated signaling? If this is the case, why is EGF also included in media cocktails optimized for this purpose? Perhaps, R-spondins and EGF act synergistically to enhance downstream signaling from EGFR?

Another question that arises from this work is whether these functions of LGR4 are conserved in the other two family members. LGR5, in particular, is one of the most common markers used to identify epithelial stem cells (10). If LGR5 also has the ability to regulate EGFR, it could change our thinking about how stem cells regulate pluripotency. The reciprocal consideration is also of interest. That is, beyond EGFR and HER2, are other receptor tyrosine kinases (RTK) also regulated by LGR4 in a similar manner. For example, MET, another member of the RTK family, is also controlled at the post-translational level by the activity of CBL (8). It will be of interest to gain further insight into the specificity of LGR4 for specific RTKs.

There is also the question of how LGR4 is able to regulate different E3 ubiquitin ligases by seemingly disparate mechanisms. In the case of RNF43 and ZNRF3, LGR4 serves as a member of an R-spondin–dependent complex that removes the E3 ligases from the cell surface. Is a similar mechanism at play for the effects of LGR4 on CBL? The authors have performed some work relevant to this question, focusing primarily on the phosphorylation of Y1068 in EGFR. However, CBL is recruited to the Y1045 upon phosphorylation, so it would be interesting to see whether LGR4 changes Y1045 phosphorylation to provide further insight into whether LGR4-mediated inhibition is due to steric hindrance of CBL enzymatic activity toward EGFR or whether other mechanisms are involved. Finally, is there cooperativity or antagonism between the ability of LGR4 to regulate EGFR and its ability to interact with RNF43/ZNRF3 and modulate FZD levels?

Like many meaningful advances, this study stimulates more questions and future research directions. Addressing these new questions may provide surprising insights that will deepen our understanding of the roles of all these proteins in carcinogenesis and offer new opportunities for therapeutic targeting.

Authors' Disclosures

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