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Review Article

Significant Role of LGR-5 in Oral Cancer and Oral Dysplasia- A Review

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***ABSTRACT*:**
Leucine-rich repeat-containing G protein coupled receptor 5 (LGR5) is a biomarker for cancer stem cell which has been responsible for the cancer development and progression. After the validation of LGR 5 as a marker of intestinal stem cells, the field has become more apparent and led to many new avenues of research. Recently, studies are also supported that LGR5 is overexpressed in diverse types of human cancers, including colorectal, gastric, esophageal, hepatocellular carcinomas and pancreatic adenocarcinoma. This emerging technology opens new possibilities of using cultured adult stem cells for drug development, disease modeling, gene therapy and regenerative medicine. This review describes the expression of LGR5 stem cells in HNSCC and summarizes subsequent progress, promises, unresolved issues, and challenges of the field.
Key words: LGR5, oral cancer, oral dysplasia.

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Introduction:

G-Protein Coupled Receptors (GPCRs) G-protein coupled receptors belong to one of the largest and most diverse families of membrane proteins. In humans GPCRs are encoded by more than 800 genes.[1] GPCRs are important signal transducers that control key physiological functions including immune responses, hormone, and enzyme release from endocrine and exocrine glands, neurotransmission, cardiac, smooth muscle contraction, and blood pressure regulation. GPCRs respond to a wide gamut of stimuli ranging from photons of light, to ions (H1 and Ca21), small organic molecules, peptides, and proteins.[2] Once ligand binding has occurred, the receptor undergoes a change that causes the activation of cytosolic signaling molecules, resulting in a cellular response. Present day drugs for allergies, hypertension, reflux, depression, asthma, and cancer all act by modulating the activity of GPCRs. In reality, 50– 60% of all current therapeutic agents directly or indirectly target GPCRs.[3] Because of their number, diversity and critical role(s) in signaling, GPCRs offer extraordinary opportunities for development of novel drugs. Defining the molecular changes that accompany function in different classes of GPCRs is not only of fundamental scientific interest, but holds enormous prospects for improving our knowledge of stem cell biology and enhancing human health. After a short introduction to the description and status of GPCR structural biology, this review focuses on a particular GPCR family, the leucine rich repeat-containing G-protein coupled receptors (LGRs).

Structure of classical GPCR family members

Structure determination of GPCRs is challenging at all stages, including protein expression, purification, and crystallization. The field is now, however, taking advantage of the high-throughput revolution in structural biology, utilizing an array of methods developed to stabilize and engineer GPCR proteins for crystallization and analysis. These methods include the introduction of T4 lysozyme and apocytochrome into linker regions of

GPCRs,[4–6] cocrystallization with simplified monoclonal antibody fragments derived from camels and llamas,[7] thermostabilization of GPCRs by multiple systematic point scanning mutagenesis[8] and protein engineering for example, introduction of non-native disulfide bridges. More standard approaches include removal of flexible portions of the receptor and use of high affinity ligands. All such approaches either reinforce crystal contacts or stabilize one conformational state over another.

The use of lipid cubic phase and other bilayer mimetic methods and the availability of new types of solubilizing detergents have further increased the crystallization potential of GPCRs. At the time of writing, 22 unique GPCR structures have been deposited in the protein database.[9] The molecular structure of a GPCR comprises three "zones" with respect to the membrane: (1) an extracellular region consisting of the N-terminus and three extracellular loops (ECL1–ECL3), (2) a transmembrane (TM) region consisting of seven a helical segments (TM1–TM7) and (3) an intracellular region consisting of three intracellular loops (ICL1–ICL3), an intracellular amphipathic helix, and the C-terminus

A detailed analysis of the different GPCR structural domains is provided in Venkatakrishnan et al.[9] Active, intermediate-active, and inactive states of GPCRs have been observed and have provided important insights into the general mechanism of GPCR activation.[10–12] The binding of ligands to the extracellular region appears to result in changes to interactions between the extracellular domain and the transmembrane region. This results in subtle conformational changes in the TM core. It is thought to precede larger structural rearrangements in the membrane cytoplasm that facilitate the binding of intracellular effectors (e.g., heterotrimeric G proteins and b-arrestins).[13]

Classification of GPCRs

Nonsensory GPCRs (i.e., those excluding light-, odor-, and taste-receptors) have been classified according to their pharmacological properties: Class A are rhodopsin-like, Class B are secretin-like, Class C are metabotropic glutamate/pheromone, and the fourth Class comprises the frizzled/smoothened receptor families. Class A is the largest and has been further subdivided into four groups a, b, g, and [14] The d group contains olfactory receptors as well as purine, MAS-related and the leucine-rich repeat-containing receptors (LGRs).

Leucine-rich repeat-containing GPCRs (LGRs)

The LGR proteins are a distinct subset of evolutionarily conserved Class A GPCRs, which harbor a rhodopsin-like GPCR and a large extracellular domain with multiple leucine-rich repeats (LRR).[15] LRRs are structural motifs that consist of a conserved 11-residue sequence rich in hydrophobic amino acids; often leucines are at defined positions (LxxLxLxxNxL, where x is any amino acid). The tertiary fold of a string of LRR repeats is known as an a=b horseshoe.[15] The extracellular domain links ligand binding to modulation of downstream LGR intracellular signaling pathways.[16] LGR family proteins have been categorized into three main groups (A, B, and C), according to the relative abundance of LRRs in the ectodomain, the presence of a low density lipoprotein receptor class A domain (LDLa) and the length of a hinge region connecting the GPCR region to the extracellular domain.[17,18]

Type A LGR receptors are characterized both by a long hinge region and by having seven to nine LRRs in their ectodomain. The glycoprotein hormone receptors, like follicle stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR), and thyroid-stimulating hormone receptor (TSHR), belong to the Type A receptor subfamily. Type C receptors have similar number of LRRs to Type A, but are distinguishable by a shorter hinge region than Type A and the presence of an LDL a motif. This subgroup includes the relaxin hormone receptors LGR7 and LGR8.[15,19] Signal transduction via Type A and C receptors is thought to occur when hormone binding to the ectodomain triggers conformational changes within the transmembrane domain, which in turn activates heterotrimeric G proteins bound to the intracellular loop. This sequence of events results in activation of downstream signaling pathways.[20]

The Type B receptor family LGR4, LGR5, and LGR6 are characterized by the presence of [13–18] LRRs within the extracellular domain. There are only three closely related proteins in this family. The LGR gene family was originally identified via in silico screens for cDNAs encoding proteins with homology to the Type A glycoprotein hormone receptor.[15,21,22] The recent explosion of interest in the LGR group of GPCRs is chiefly due to the their presence on the epithelial stem cells of hair, skin, intestine, and breast tissues.[23–27]

Discovery and Validation of LGR5 as Adult Stem Cell

Marker LGR5 is a Wnt target gene[28] and was discovered by researchers trying to find an interstitial stem cell marker.[29] It has been known for many decades that the intestinal epithelium regenerates constantly[23] and a small population of stem cells residing at the base of the intestinal crypts drives this regeneration process.[30] However, the identity of the crypt stem cells remained elusive because of a lack of specific markers. Epithelial homeostasis in the adult intestine is orchestrated by several signaling pathways including EGFR,[31] EpH,[32] Notch,[33] Hedgehog,[34] and Wnt.[35] Wnt signaling plays a critical role in maintaining intestinal epithelial cell proliferation.[35] Hyperactivation of the Wnt pathway is associated with adenomatous transformation of the intestinal epithelium [36] [similar to adenomatous transformation caused by loss of the tumor suppressor gene, adenomatous polyposis coli (APC) [36]] and is the principal cause of colon cancer in humans.[37,38] The role that Wnt signaling plays in the physiology of the intestine suggested that one or more Wnt target genes could be stem cell markers. Clevers and coworkers identified a Wnt driven genetic programme that is activated in APC-mutant human colon cancer cells.[29]

The expression programme consists of core set of 80 genes. Although the majority of these genes are expressed

throughout the proliferative crypt compartment [28,29] and in mature Paneth cells,[39] the expression of several Wnt target genes appeared to be restricted to the base of the crypts, that is, the stem cell compartment. Of the basally expressed genes, LGR5 is specifically expressed in small wedged-shaped cells present in between the Paneth cells at the base of the small intestinal crypts. These wedged-shaped cells are known as "crypt base columnar" (CBC) cells and had been identified in 1974 by Cheng and Leblond using electron microscopy.[40] CBC cells are morphologically immature cells that gained prominence as a candidate stem cell population following the publication of the "stem cell zone" model by Bjerknes and Cheng.[41] LGR5 has now emerged as a candidate stem cell marker in the intestinal crypts. Further examination of LGR5 expression patterns in the mouse found discrete populations of LGR5 expressing cells (LGR51) in other organs, including skin, large intestine, stomach, mammary gland, tongue, kidney, and endometrium,[23–25,42–46] suggesting that LGR5 is a potential "universal epithelial stem cell marker."[44,47] To validate the LGR51 population as adult epithelial stem cells, in vivo lineage-tracing experiments were conducted on LGR5-expressing CBC cells in mouse small intestine.[23] In vivo lineage tracing is a genetic fate-mapping technique in which heritable genetic marks are introduced into candidate stem cell populations in situ in living tissues.[48]

The descendants of these marked stem cell candidates can be probed in situ for the introduced genetic markers.48 A marked stem cell candidate is said to be multipotent if the entire set of differentiated cell lineages can be traced back to a single stem cell and long-term production of marked cell lineages in a given tissue exhibits the self-renewal capacity of the stem cell candidate.48 Thus a candidate cell demonstrating both multipotency and self renewal capacity in this system fulfills the requirements to be called an adult stem cell (possessing "stemness").[48] To evaluate the "stemness" of LGR51 populations in vivo using lineage tracing, a heritable-inducible lacZ reporter gene was introduced into LGR5expressing cells. Initially resulting in the appearance of lacZ1 cells in the CBC compartment within the crypt base,[23] over the course of the week the progressively expanding lacZ1 progeny were observed extending from the crypt base towards the tips of interstitial villi. Similar observations were also made in colon.[23] Thus, individual lacZ1 tracing units were present in all epithelial cell lineages and persisted throughout the life of the organism, identifying LGR51 cells as a truly multipotent, self-renewing population of adult intestinal stem cells. In vitro, small numbers of LGR1 cells are able to generate self organizing, self-renewing epithelial organoids with an architecture and cell composition that are remarkably similar to in vivo crypts/villus units.[49] In vivo and in vitro data identify the LGR51 cells in the mouse intestine as the proliferating stem cells responsible for the daily self-renewal capacity of the mucous lining. In vivo lineage tracing has also been used to demonstrate "stemness" of LGR5expressing populations in the adult hair follicle, adult distal stomach, taste buds, and embryonic kidney.[24,25,42,43,46] Recently it was shown that mammary glands can be reconstructed efficiently fromLGR51 cells.[45] These reconstructed mammary glands exhibit regenerative capacity in serial transplantations.45 Adult tissues that display lower turnover rates, such as the liver,[50] respond to acute damage by activating Wnt signaling and consequentially generate LGR51 stem cells that result in tissue regeneration.[51]

Mechanism of maintaining epithelial cell homeostasis by LGR5+ stem cells

Validation of LGR5 as a stem cell marker of intestinal epithelial cells allowed the role of stem cells in homeostasis to be studied in greater depth. The stem cell-driven process that maintains the homeostasis of continually renewing intestinal epithelia requires a delicate balance between daily production of committed progeny and new stem cells throughout the lifetime of an organism. Understanding this process in the adult stem cell compartment in vivo is crucial for deciphering how disturbance to this equilibrium contributes to disorders such as cancer. It has been proposed that adult stem cells within tissues undergo obligate asymmetric division to maintain the balance between production of committed progeny and new stem cells.[52] However, recent studies have found compelling evidence of prevalently stochastic, symmetric cell division within the LGR51 stem cell compartment. In particular, multicolor lineage tracing experiments show that cell division in LGR51 stem cells is symmetric In the short-term, LGR51 stem cells rarely generate daughter cells that adopt divergent fates. In the long-term, however, the multicolor stem cell pool is converted to a single-color population, indicating a gradual shift towards clonality.[53] Thus it appears likely that LGR51 stem cells double daily and that adoption of stem cell or progenitor fate is determined stochastically. It has been independently demonstrated that the segregation of chromosomes during mitosis of LGR51 intestinal stem cells is random. At present the molecular mechanisms that stimulate LGR51 intestinal stem cell division and their subsequent fate are not known.

Functions and mechanism of action of LGR5

Much of our understanding of LGR5 function has come from the analysis of null or loss-of-function mutants. A knock-in mouse strain harboring a lacZ reporter gene 50 to the region that encodes the first transmembrane domain creates a null allele.[54] In homozygotes, disruption of LGR5 results in 100% neonatal lethality, characterized by gastrointestinal tract dilation and absence of milk in the stomach. Histological examination of the homozygote mice revealed fusion of the tongue to the floor of the oral cavity (condition called ankyloglossia), while immunostaining showed expression of LGR5 in the epithelia of the tongue and mandibles of wild-type embryos. Thus, neonatal lethality of the LGR5 null mice provided the first firm indication that LGR5 is essential in development. The same LGR5-null strain also demonstrated accelerated maturation of Paneth cells in the developing intestine, indicating that LGR5 may negatively regulate Wnt signaling during neonatal

intestinal development.[55] Further evidence that LGR5 negatively regulates Wnt signaling has also been indicated in colorectal cancer cell lines by overexpression of LGR5 or reduction of LGR5 expression by RNAi.[56] Walker et al. illustrated that overexpressing LGR5 in a colon cancer cell line suppresses the response to Wnt signaling, augments cell–cell adhesion, reduces clonogenicity and attenuates tumorigenicity.[56] Conversely, knockdown of LGR5 resulted in enhancement of Wnt signaling attributes such as increased invasion, anchorage independent growth, and enhanced tumorigenicity.[56]

R-spondins are ligands of LGR5 In 2011, it was discovered that R-spondin (RSPO) family proteins were ligands of LGR5.[57–61] Rspondins are required for the production of crypts in vivo and in vitro [49] and have a strong mitogenic effect on LGR51 cells.[62,63] The interaction of RSPOs and LGR5 have been assessed by cell surface binding assays, surface plasmon resonance, cell-free coimmunoprecipitation, and a tandem affinity purification mass spectrometry.[57–59] The Kds of binding between different RSPOs and LGR5 are in the nanometer range, (e.g., the Kd of hRSPO1-LGR5 interaction was measured at 3.1 nM [57,58] and that Kd of RSPO3 and LGR5 3.0 nM).[59] R-spondins are secreted proteins of 35 kDa and RSPO1-RSPO4 share pair-wise amino-acid similarity of 40–60%. The human RSPO1–4 proteins range from 234 to 272 amino acids in length and feature: (i) a hydrophobic, secretion signal sequence at the N-terminus, (ii) adjacent cysteine-rich furinlike (FU) repeats, (iii) a thrombospondin Type I repeat (TSR) domain that can bind matrix glycosaminoglycans and/or proteoglycans, and (iv) a C-terminus basic amino acid-rich (BR) domain of varying length (Fig. 2). Although RSPOs do not initiate Wnt signaling, they bind LGR5, and presumably release its negative regulation of Wnt signaling, thus potentiating Wnt signaling.[58,59,64–66]

Implication of LGR 5 in oral dysplastic and oral squamous cell carcinoma

Cancer stem cells (CSCs) were originally identified in acute myeloid leukemia. At later, CSCs were found in various other malignancies, such as lung, colon, breast, ovary, stomach, and liver cancers.[5,7,12,20,23,24] CSCs play an important role in the process of initiation, development, metastasis, immune evasion and recurrence of cancers.[8,25] OSCC is a highly heterogeneous cancer. For avoiding any intratumoral heterogeneity of biomarker expression, we chose ten HPF representative fields from different areas of every OSCC's section to analyze immunostaining results. LGR5 is a common biomarker of CSCs and was expressed at the base of crypt stem cells. In this study, we analyzed LGR5 protein expression in OSCC and corresponding normal oral cavity mucosa tissues from 190 patients and compared to clinicopathological characteristics. We found that LGR5 expression was significantly higher in OSCC tissues than that in the normal tissues. Moreover, it was positively associated with tumor size, grade, LNM, and TNM stages. Furthermore, Kaplan-Meier survival analysis suggested that OSCC patients with LGR5-positive expression had significantly shorter survival time than did LGR5-negative patients. Our findings are similar to the other studies demonstrating that LGR5 should be effective as clinical biomarker for OSCC.[8,26,27]

Angiogenesis supports the rapid growth of tumor by its functions of transporting nutrient and oxygen. The traditional angiogenesis theory was focused on the endothelial cells forming the neovasculature from preexisting vascular. However, the clinical benefits of anti-angiogenesis for cancer therapy is still unsatisfactory.[15,28]

This may indicate that there is another mechanism of tumor blood supply. In 1999, Maniotis and his coworker found a new blood supply which directly interconnected to form channel-like structures by tumor cells-vasculogenic mimicry (VM).[16] Accumulating evidence suggested that VM plays an important role in promoting blood supply for tumors. Results in this study demonstrated that positive rate of VM was significantly higher in OSCC samples than that in the control samples and its positive rate was positively associated with tumor size, grade, LNM, and TNM stages. Moreover, we found that patients with positive VM had significantly lower survival time than did VM-negative patients. The above findings suggested that VM should be involved in the progression and metastasis of OSCC, and could be an effective biomarker in conducting this dis ease. Our results are similar to previous studies, including those of OSCC and other malignancies.[20, 28-31]

TNM stages can provide guidelines therapeutic tactics for patients with OSCC, however, it can't provide entire information about OSCC's bio logical behavior. Therefore, it is urgent to find novel and efficient biomarker to predict OSCC's patient biological behavior. In this study, multivariate analysis suggested that LGR5 expression, positive VM, LNM, as well as TNM stages are independent prognostic biomarkers for OSCC patients. This finding demonstrates that LGR5 and VM should be considered as credible biomarkers for OSCC, especially in predicting prognosis.

The niche where CSCs reside is composed of microvessels and microlymphatic vessels. Vascular niche can regulate CSCs self-renewal. CSCs can promote angiogenesis to meet rapid tumor growth.[32] CSCs can differentiate various differentiation tumor cells and stromal cells, including endothelial cells.[33] So CSCs can mimic endothelial cells to form tube structures---VM in the tumor tissues. In this study, there was a positive association between the positive expression of LGR5 and VM in OSCC. This indicated that CSCs and VM should promote OSCC's proliferation, progression, and metastasis.

Conclusion

Our article imply that LGR5 affect OSCC metastasis and prognosis, and combined detection of LGR5 and VM, to some extent, should reflect OSCC's cell biological behavior, thus considering as valuable biomarkers of metastasis and prognosis in OSCC.

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