miR-17-92/p38α dysregulation enhances Wnt signaling and selects Lgr6+ cancer stem-like cells during lung adenocarcinoma progression

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Running Title

Lgr6 labels malignant human adenocarcinoma cells.

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Guinot et al.,

ABSTRACT

Defining the molecular and cellular roots of lung cancer relapse after initial treatment remains an imperative to improve survival. Here we report that the lung stem cell marker Lgr6 becomes enriched in non-small cell lung cancer (NSCLC) cells during malignant progression. Lgr6+ NSCLC cells displayed self-renewal and differentiation properties along with a higher tumorigenic potential. Mechanistic investigations suggested that a defective repression of the miR-17-92 gene cluster was responsible for evolution of a selection for outgrowth of Lgr6+ NSCLC cells. High levels of expression of miR-19 family members were found to target and downregulate levels of p38α kinase, providing a specific survival signal for Lgr6+ cells as mediated by increased Wnt/β-catenin activity. Our results identify a specific stem-like cell population in NSCLC with increased malignant potential, the elucidation of which may enable earlier prognosis and possibly the development of more effective targeted treatments.
INTRODUCTION

Lung cancer is the major cause of cancer related deaths in western countries (1). Early detection and surgery have proven to be the most effective ways to improve survival (2). Important advances have been made in the last 20 years, with the detection of oncogenes and mutations linked to many lung cancer types. However, therapies applied using drugs directed to those molecules or related pathways have failed to significantly increase survival, especially in the most prevalent cancer types, e.g. lung adenocarcinoma (3-5). Cancer relapse after radio- and chemo-therapy is the essential reason for reduced survival (6). Previously, efforts have focused on investigating the cells and mechanisms involved in the resistance and later virulence of lung cancers after therapeutic treatments (7, 8). Recent theories in cancer research consider the existence of cell populations with stem cell like properties in many cancer types (9, 10). These cancer stem cells would be responsible for tumor maintenance and would harbor the potential to colonize other tissues as metastatic cells (11, 12). These cells would also be especially resistant to drugs, and they would be selected during tumor progression, generating more aggressive and metastatic disease (13, 14).

In this paper, we have studied the cellular and molecular mechanisms behind the development and progression of human lung adenocarcinoma. We have previously characterized a population of human lung cells with stem cell potential to differentiate into all bronchioalveolar lineages while being able to self-renew. These epithelial cells express the stem cell marker Lgr6 (Leucine-rich repeat-containing G protein-coupled receptor 6). Lgr6, and its homologous receptors, are amplifiers of Wnt signaling and are involved in maintaining stem cell self-renewal (15). We have analyzed the contribution of Lgr6+ cells to human lung adenocarcinomas. We have observed that cells expressing Lgr6 are enriched from early to late stages of human lung adenocarcinomas malignancy.
These cells showed a disruption in the balance between the p38α and miR-17-92 pathways. p53 mutation prevents its repression of miR-17-92 expression (16). Crosstalk between signaling pathways and its role in cancer has been extensively reported elsewhere (17, 18). Reduced p38α promotes activation of Wnt signaling and, in human lung cancer samples, we observed a correlation between a deficiency in p38α protein and increase of Wnt signaling, promoting Lgr6+ cells during cancer progression. These findings provide a new tool for early prognosis of human lung adenocarcinomas. We demonstrate how a novel specific population of cells, and their regulatory mechanisms, is linked and contribute to better understanding of the malignant progression of lung adenocarcinomas. This co-adjuvant process, in parallel to oncogenic mutations, helps select cells with a higher potential to perpetuate (self-renewal) and manipulate the microenvironment, facilitating colonization of foreign tissues (metastasis).

MATERIAL AND METHODS

Isolation and culture of human lung adenocarcinoma cells

Cultures were established from freshly isolated lung adenocarcinoma cells obtained from cancer patients undergoing tumor resection (Papworth hospital, UK). Approval by the Ethics Committee at Cambridge University and the Papworth Hospital was received. All patients gave their informed consent. Papworth Hospital pathologists provided information related to the type, stage and grade of the tumors. Human lung adenocarcinomas specimens were finely minced and resuspended in a mix of 1 mg/ml collagenase type 1/collagenase type 3 (Worthington Biochemical, Cat. No: LS004196 and LS004182) in DMEM. The suspension was incubated at 37°C with shaking (220 rpm) for 45 minutes. The suspension was centrifuged for 5 minutes at
1200 rpm, and the pellet was resuspended in fresh DMEM before filtration through a 70 μm cell strainer (BD Falcon, Cat. No: 352350). After a second centrifugation (5 minutes at 1200 rpm), the pellet was washed in PBS and treated with red blood cell lysis buffer (Roche, Cat. No: 11814389001) for 5 minutes. The cells were pelleted again (5 minutes at 1500 rpm), washed in PBS, filtered through a 50 μm CellTrics sterile filter (Partec, Cat. No: 04-004-2327) and placed in culture. Cells were cultured in 1:1 DMEM medium (Gibco, Cat. No: 41965-039) and Ham’s F12 (Lonza, Cat. No: BE12-615F) supplemented with 5% FBS (Gibco, Cat. No: 10270-106), hEGF (10ng/ml, Pepro Tech, Cat. No: AF-100-15), FGF-2 (20ng/ml, Pepro Tech, Cat. No: 100-18B) and 0.1% Pen/Strep (Sigma, Cat. No: P0781) antibiotics. Cells were incubated at 37°C in 93% air with 7% CO2. Medium was changed every 2 to 3 days. Cells were disaggregated using accutase (PAA, Cat. No: L11-007) for 5 minutes at 37°C to produce a single cell suspension. Cells were then pelleted in a centrifuge (1200 rpm for 5 minutes), washed in PBS (Sigma, Cat. No: D8537), pelleted a second time and resuspended in fresh medium.

All cells used were directly isolated from human samples and characterized by immunohistochemistry and qPCR expression of lung and adenocarcinoma markers. No commercial cell lines were used for this work.

**p38α siRNA knock-down**

Lgr6+ human lung stem cells’ isolation and culture has been previously described by our group (16).

Lgr6+ p38α knock-down cells (Lgr6+ p38KD) were obtained by infecting Lgr6+ human lung stem cells with lentiviral vector pLKO.1-TRC (Sigma, Cat. No: SHC001) carrying an shRNA construct to knock-down p38α (5’-CCCGT-
GTCCATCATGCAGAAA-TTCAAGAGA-TTTCGCATGAATGATGGACTG-TTTTTT-G) and a puromycin selection marker. Cells were selected in puromycin (15 μg/ml, Sigma, Cat. No: P9620) for 10 days.

Lgr6+ p38a knock-down cells with miR-17-92 rescue (Lgr6+ p3KD shmiR) were obtained by infecting Lgr6+ p3KD cells with lentiviral vector pLKO.1-TRC carrying an shRNA construct to knock-down pri-miR-17-92 (5'-CCGGT-AAGGAGAGCTCAATCTGCACA-CTCGAGTG-TGCAGATTGAGCTCTCC-TTTTTT-G) and a geneticin selection marker. Cells were selected in G418 (0.5 mg/ml, Invitrogen, Cat. No: 10131019) for 10 days.

In both cases infectious virus was added to cells in the presence of 8 mg/ml polybrene (hexadimethrine bromide, Sigma) and incubated for 6 h.

**Histology and immunostaining**

Lung adenocarcinoma tissue samples and xenografts were fixed overnight at 4°C in 4% paraformaldehyde (PFA) and embedded in paraffin or OCT. Paraffin blocks were cut in 5 μm sections using a microtome. OCT blocks were cut in 10 μm sections using a cryostat microtome at -20°C. H&E staining was performed using a Leica Autostainer XL.

Primary antibodies used: anti-human Lgr6 (Santa Cruz, sc-99123), anti-human TTF1 (Abcam, ab-40880), anti-human Ki67 (Vector, VP-RM04), anti-human p53 (Novocastra, P53-CM5P), anti-human P-GSK3 (Ser9) (Abcam, ab-75814), anti-human β-catenin (Santa Cruz, sc-7199), anti-human mitochondria (Thermo Scientific, MA5-12017), anti-human SP-C (Santa Cruz, sc-7705), anti-human CC-10 (Santa Cruz, sc-25554), anti-human E-Cadherin (Abcam, ab-53033), anti-human CK7 (Abcam, ab-90083), anti-human p63 (Santa Cruz, sc-8431).
The Ventana Discovery (Ventana Medical Systems) automated system was used for immunohistochemistry (IHC) on paraffin and frozen sections. Ventana Cell Conditioning 1 solution (CC1) was used to perform antigen retrieval in all sections. Antibody detection was performed using the DAB Map detection kit (Ventana, Cat. No: 760-124), sections were counterstained with haematoxylin using the Leica Autostainer system and mounted manually.

For immunofluorescent staining, sections were incubated in blocking buffer (PBS, 4% donkey serum, 1% Triton) for 1 h at room temperature. Primary antibodies were incubated overnight at 4°C. Sections were rinsed three times in PBS and incubated with secondary antibodies diluted at 1:1000 for 1 h at room temperature. Slides were mounted in Vectashield® mounting media with DAPI.

**Immunostaining quantification**

The number of positively stained cells on sections was quantified using Cell Profiler software. Three or more biological replicates of different stages of human lung adenocarcinoma were used to obtain the average number of positive cells in randomly selected fields. The significance of quantitative data was tested using an unpaired, two-tailed t-test. The standard deviation of the mean is represented in the graphs by error bars.

**Western blotting**

Cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% (v/v) NP-40, 5 mM EDTA pH 8.0, 5 mM EGTA pH 8.0, 20 mM NaF, 0.1 µM PMSF, 0.1 µM NaVO₃, plus complete protease inhibitor cocktail (Roche) and cellular lysates were separated by SDS-PAGE and transferred to PVDF membranes. The following
antibodies were used for protein detection: p38α MAPK (Cell Signaling, #9228), Lgr6 (Santa Cruz, sc-99123), α-tubulin (Sigma, T9026), P-GSK3 (Cell Signaling, #9323).

**Total RNA isolation and quantitative RT-PCR.**

Total RNA was extracted from cells or tissue using miRVana miRNA isolation kit (Ambion, Cat. No: AM1560) following the manufacturer’s instructions and was treated with DNAse I (Promega, Cat. No: M6101). MicroRNA expression was detected using TaqMan microRNA assays: U6 snRNA (001973), hsa-miR-17a (002038), hsa-miR-18a (002422), hsa-miR19a (000395), hsa-miR-19b (002425), hsa-miR-20a (000580), hsa-miR-92a (000431).

Expression of all other genes was quantified using SYBR Green technology and normalized to a housekeeping gene (GAPDH or HPRT), using specific primers (Table 1). Specificity of PCR products was tested by dissociation curves.

**Flow cytometry**

Single-cell FACS (MoFlo, Beckman Coulter) was performed following one hour incubation with anti-Lgr6 (Santa Cruz, sc-99123) and one hour in the dark with anti-Rabbit PE secondary antibody (Santa Cruz, sc-3745).

Flow cytometry analysis was performed (BD LSRFortessa) following fixation in 2% PFA and incubation in anti-Ki67 (Vector, VP-RM04) and anti-Lgr6 (Santa Cruz, sc-48236). Cells were incubated in the dark for one hour with the following secondary antibodies: anti-Rabbit Alexa Fluor 488 (Invitrogen, A21206) and anti-Goat Alexa Fluor 555 (Invitrogen, A21432).

**Soft agar colony formation assay**
A 1% low melting agarose (Bio-Rad, Cat. No: 161-3111) aliquot was melted in a microwave and subsequently cooled down to 40°C in a water bath. DMEM 10% FBS, pre-warmed at 40°C, was used to produce a final concentration of 0.5% agarose by mixing equal volumes of both. A base layer of 1 to 2 ml of 0.5% agarose was placed at the bottom of each well of a 6-well plate (BD Falcon, Cat. No: 353046) and allowed to cool down for 2-3 minutes at 4°C and then at room temperature. Different amounts of single cells were resuspended in DMEM 10% FBS and mixed equally (1:1) with 40°C 0.7% agarose to produce a final concentration of 0.33% agarose. Carefully, 1.5 ml of the cell mixtures were pipetted on top of each base layer and allowed to settle for 30 minutes at room temperature. Cells in this semi-solid media were incubated at 37°C for 3 weeks and then stained with 0.005% crystal violet solution to allow colony counting.

**Cell proliferation assay**

In 24-well plates, 4x10^5 cells per well were plated in triplicates in general culture conditions. The number of viable cells was counted every day for 6 days using an automated Vi-CELL cell counter (Beckman-Coulter, Cat. No: 731050).

**Mouse experiments**

All mouse experiments were subjected to ethical approval and performed according to United Kingdom Home Office Regulations. CD-1 nude and NOD/SCID mice (Charles River) were maintained under standard pathogen-free conditions.

**Subcutaneous injections**

Freshly isolated lung adenocarcinoma cells were resuspended in 100 μl of a 1:1 mix of culture medium and matrigel (BD, Cat. No: 356230) following Lgr6 FACS sorting.
Cells were injected under the skin of 6 to 8 week old nude or NOD/SCID mice with a 0.5 ml syringe. Mice were killed 10 to 12 weeks later. Subcutaneous growths were removed and prepared for histology sections.

**Tail vein injections**

NOD/SCID male mice were warmed for a few minutes, to induce vasodilatation, in a heated IVC recovery chamber set at 40°C. Mice were then put in a restraint tube and the tail cleaned using warm surgical scrub and water. A single-cell suspension of freshly isolated lung adenocarcinoma cells following FACS sorting, was resuspended in 100 μl PBS following FACS sorting and injected into the tail vein using a 30G needle and 1 ml syringe. Mice were killed 10 to 12 weeks later. Lungs were removed and prepared for histology sections.

**RESULTS**

We have described a population of human lung cells with the potential to differentiate both, *in vitro* and *in vivo*, into all mature bronchioalveolar cell types. These cells specifically express the membrane marker Lgr6 (16). In mouse lung cancer models, bronchioalveolar stem cells are expanded during adenocarcinoma transformation (19, 20). We studied the expression of the human bronchioalveolar stem cell marker Lgr6 in human lung adenocarcinomas. The adenocarcinoma origin of the tumors was confirmed as TTF1+/p63- to discard any tumors of squamous origin (Suppl. Fig. 1).

**Accumulation of Lgr6 positive cells during human lung adenocarcinoma progression**

We have analyzed 53 human lung cancers for the expression of a number of lung specific, cancer, and stem cell markers in samples of lung small cell, squamous and
adenocarcinoma at different stages. We observed that adenocarcinomas (TTF-1 positive) showed increased expression of the stem cell marker Lgr6 at later stages (Fig. 1a). Lgr6 expression in both earlier (Stage 1, Stage 2) or later (Stages 3, 4 and metastatic) disease was specifically localized to the tumor area, being absent from the surrounding healthy tissue (Fig. 1a). There was a direct correlation between adenocarcinoma stage and Lgr6 expression levels in tumor samples, as observed by protein expression (Fig. 1b). Analysis of cell populations from different stage adenocarcinomas by flow cytometry confirmed the increase in the Lgr6+ population during tumor progression and was shown to be particularly enriched after Stage 2 (Fig. 1c). In order to further understand how Lgr6+ cells are selected, we screened early and late stage adenocarcinomas for both proliferation and apoptosis. Immunostaining of tumor samples revealed an increase of the proliferation marker Ki67 at later stages (Fig. 1d). Analysis by TUNEL staining could not detect any difference in apoptosis at any stage (data not shown). Ki67+ cells at Stage 2 are mostly Lgr6+ cells (Fig. 1e). Thus, there is a proliferative advantage that may contribute to specifically select Lgr6 cells during adenocarcinoma progression.

Selection of Lgr6 cells with a disrupted p38\(\alpha\)/miR-17-92 axis

We have previously shown that human lung Lgr6+ stem cells are regulated by a fine tuned regulated crosstalk between the p38\(\alpha\) MAPK pathway and the microRNA cluster miR-17-92 (16). In addition, miR-17-92 has been reported to be overexpressed in lung tumors (21, 22). Analysis of miR-17-92 expression by qPCR in tumors from different adenocarcinoma stages confirmed the increased expression of the cluster members during cancer progression (Fig. 2a). We have previously shown that expression of the miR-17-92 cluster is transcriptionally repressed by p53 (16). Analysis of p53 by
immunohistochemistry showed that nuclear p53 is lost during adenocarcinoma progression correlating with higher miR-17-92 levels (Fig. 2b). In addition, enhanced miR-17-92 expression correlated with decreased levels of p38α in late stage adenocarcinomas, when compared to their early stage counterparts (Fig. 2c). p38α protein, but not mRNA expression, has been shown to be reduced in human lung tumors (20). Hence, downregulation of p38α must be a post-transcriptional event. Using public databases (www.targetscan.org), we noted that p38α was a putative target for one of the miR-17-92 members, miR-19. Using a lentiviral vector in cultured lung Lgr6+ cells, we observed that miR-19 overexpression induces proliferation (Suppl. Fig. 2a, b). miR-19 specific targeting of p38α 3’-UTR was shown using a luciferase reporter assay (Suppl. Fig. 2C), and results in decreased p38α protein (Fig. 2d). This is a previously unreported negative crosstalk regulation and it creates a negative feedback loop between the miR-17-92 and p38α pathways (Suppl. Fig. 2d).

Repression of p38α is enhanced in Lgr6+ cells from Stage 2 tumors, when compared to counterpart Lgr6- cells (Fig. 2e). Reduced p38α protein in Lgr6+ correlates with a change in the expression of miR-17-92 members, larger than its increase in Lgr6- cells, during cancer progression (Fig. 2f).

**p38 regulates the expression of Wnt signaling components**

Wnt signaling is a key pathway related to cancer development is (23). Inhibitory phosphorylation of GSK3 and nuclear translocation of β-Catenin are hallmarks of canonical Wnt signaling and are present in many cancers (24, 25). We observed increased P-GSK3 levels with increasing lung adenocarcinoma stage (Fig. 3a). Activation of the canonical Wnt pathway and enhanced signal was confirmed by immunohistochemistry, showing specific P-GSK3 and nuclear β-Catenin induction in
late stage lung adenocarcinomas (Fig. 3b). Further confirmation was established by qPCR analysis of mRNA expression of Wnt components with elevated levels of enhancers (Lrp6) and targets (Lef1, Tcf4, Axin2) and reduced levels of Wnt repressors (Wif1, Gremlin, Dkk1, Dab2) in late stages (Fig. 3c and Table 1).

Lgr6 is a promoter of Wnt receptor signaling. The co-activator Lrp6 interacts with Lgr6 enhancing the response to Wnt ligands, amplifying the receptors signal (26, 27). Activation in of Wnt signaling was analyzed in Lgr6 tumor cells. qPCR analysis of Wnt components in Lgr6+ or Lgr6- cells from Stage 2 or Stage 3 adenocarcinomas showed enhanced Wnt signaling in later stage adenocarcinomas (Fig. 3d and Suppl. Fig. 3).

Using inactivating phospho-GSK3 (Ser9) as a sensor of Wnt signal, we observed that normal lung Lgr6+ cells, deficient in p38α (p38KD), showed an increase in P-GSK3. This is independent of miR-17-92, as combined downregulation of this pathway (p38KD/shmiR) failed to rescue Wnt activation (Fig. 3e). Loss of p38α modified the expression of other components of the Wnt pathway. Interestingly, p38α-deficient Lgr6+ lung cells showed an increased expression of the Wnt co-receptor Lrp6 and decreased of the Wnt antagonist DKK1 (Fig. 3f). Inactivation of GSK3 correlates with augmented expression of genes regulated by canonical Wnt, confirming the enhanced activation of the pathway in Lgr6+ cells lacking p38α (Fig. 3g). The specific role of p38α as a negative regulator of canonical Wnt signaling in Lgr6+ cells was confirmed by overexpression of p38α in Lgr6+/p38KD cells, restoring the P-GSK3 to control levels (Fig. 3h). Partial rescue, by p38α overexpression, was confirmed by qPCR analysis with significant reduction of Wnt targets and increase in Wnt repressors (Fig. 3i).

**Selected Lgr6 cells have cancer stem cell like properties**
Activation of Wnt signal may account for maintaining Lgr6+ cells in an undifferentiated state whilst retaining stem cell features (28, 29). Lung adenocarcinomas showed a loss of lung (Suppl. Fig. 1b) and epithelial markers (Suppl. Fig. 4a, b, c), while increasing Lgr6+ cell numbers in later malignant stages (Suppl. Fig. 4e).

To test some of the tumorigenic and stem-like properties of the Lgr6+ cells, we sorted cells from different stages of lung adenocarcinoma. Both Lgr6- and Lgr6+ tumor cells express lung markers at similar levels lung markers (Fig. 4a). Interestingly, only the marker AQP5 (aquaporin 5) is overexpressed in Lgr6+ tumor cells. This protein is a marker for lung cancer malignancy and has been reported that overexpression links lung cancer with tumor invasion, proliferation and metastatic features (30, 31).

In culture, cells from later stages showed an increase in proliferation, expanding at higher rates (Suppl. Fig. 4d). As Stage 2 seemed to be the switching point where Lgr6+ cells start to prevail, we used tumor cells from adenocarcinomas at this stage to compare the properties of Lgr6+ and Lgr6- cells. A widely used in vitro assay to test the tumorigenic capacity and self-renewal potential of cancer cells is colony forming potential. Freshly isolated Lgr6+ and Lgr6- cells from Stage 2 adenocarcinomas were seeded at low confluency (10^5). Only Lgr6+ cells produced visible macroscopic colonies, confirming a higher tumorigenic and self-renewal capacity (Fig. 4b).

Lgr6+ and Lgr6- cells from Stage 2 adenocarcinoma were tested in vivo by intravenous (IV) injection into immunodeficient mice. This procedure has been extensively used to deliver cancer cells into the lungs, allowing the secondary tumor potentiality of the cells to be investigated. Similar to the in vitro assays, we only observed tumor burdens in the lungs of mice injected with Lgr6+ cells. We failed to detect any tumors from Lgr6- cells. Newly formed lung tumors from Lgr6+ cells were usually located close to blood vessels and their human lung adenocarcinoma origin was confirmed by staining with
Guinot et al.,

TTF1 and a human specific mitochondrial antibody (Fig. 4c). Interestingly, Stage 2 Lgr6+ tumor cells retained differentiation potential. The tumor masses formed from injected Lgr6+ human tumor cells were only partially positive for Lgr6 whereas they expressed lung specific markers such as SP-C or CC-10 (Fig. 4d). This confirms a putative cancer stem cell role for Lgr6+ human lung adenocarcinoma cells. We recapitulated the tumors formed by Lgr6+ cancer cells using normal Lgr6+ lung cells transformed by overexpression of oncogenic K-RasG12D. These cells co-expressed GFP and could be visually traced (green) in the lung tumors (Suppl. Fig. 5a).

Histological analysis showed features similar to those from Lgr6+ adenocarcinoma cells (Suppl. Fig. 5b), and they expressed GFP and the adenocarcinoma markers TTF1 and CK7 but not the squamous carcinoma marker p63 (Suppl. Fig. 5c). Immunofluorescent staining confirmed the human origin of the tumors formed by Lgr6+ transformed lung cells and the adenocarcinoma features, as they expressed TTF1 (Suppl. Fig. 5d).

Then in vivo tumor colonizing potential of human cancer cells was tested using subcutaneous injections into immunodeficient mice. This method assesses the capacity of the tumor cells to grow new burdens in an alien environment, one of the hallmarks of metastasis. We isolated Lgr6+ and Lgr6- tumor cells from early (Stage 1), medium (Stage 2) and late (metastatic) human lung adenocarcinomas. Freshly isolated cells from single tumors were injected in parallel in both flanks of single mice (Fig. 5a). As has been previously observed in IV injections, Lgr6- cells failed to form subcutaneous tumors. Tumors from Lgr6+ cells were histologically analyzed (Fig. 5a). The human lung adenocarcinoma origin of the resected tumors was confirmed, with expression of TTF1 and human mitochondrial antigen evident (Fig. 5b). Further analysis by immunofluorescent staining revealed the loss of epithelial (E-Cad) and the gaining of stem cell (Lgr6) markers during the progression from early to late stage disease (Fig.
Lung differentiation was also affected. Early stage tumors showed expression of alveolar (SP-C) and bronchiolar (CC-10) markers, with only marginal expression of these markers in late tumors (Fig. 5c). The lack of cellular differentiation within the tumor correlated with the observed increase in Wnt signaling and deficiency in p38α observed in Lgr6+ cells.

Loss of p38α during lung adenocarcinoma progression favors the selection of Lgr6+ stem cell-like cells with reduced differentiation potential while maintaining their colonizing and self-renewal capabilities. This changes result in tumors containing poorly differentiated cells with a high metastatic potential.

**DISCUSSION**

Despite all of the advances in unraveling the molecular and biological initiation and progression of cancer (32, 33), the knowledge of these processes in lung adenocarcinoma is still poor, resulting in little improvement in patient survival (5).

In this manuscript, we revealed the potential of a population of human lung adenocarcinoma cells, characterized for expressing Lgr6, that are selected through the progression of the cancer and that harbor cancer stem cell like properties. As a consequence of p53 mutating inactivation, there is a dysregulation of the balance between two pathways, the miR-17-92 family of microRNAs and the p38α kinase, that control normal homeostasis in human Lgr6+ lung stem cells. Lack of p53 leads to loss of its repressive effects on miR-17-92 cluster expression. miR-17-92 overexpression is a common feature in lung cancer. We have now shown that a miR-17-92 member, miR-19, downregulates p38α.

We have previously reported a deficiency in p38α protein in human lung cancer (20). There is some evidence of an inhibitory role of p38α on Wnt signaling (31, 34). Here
we have found that p38α deficiency results in a wide-ranging dysregulation of Wnt components, repression of Wnt inhibitors and promotion of its enhancers. Wnt signaling is a regulator of stem cell self-renewal and a signature pathway in many cancers (15). One of the components overexpressed in the absence of p38α is the Low-density lipoprotein-related receptor 6 (LRP6), which plays a central role in canonical Wnt signal (26, 35). We have demonstrated the specificity of this p38α function, as it can be rescued by recovering p38α expression. LRP6 function can be enhanced by interacting with R-spondin receptors like Lgr6 (27). Cells expressing Lgr6 can respond to Wnt activation, enhancing the canonical signal and, hence, promoting growth. Lgr6+ adenocarcinoma cells proliferate more than Lgr6- as the cancer progresses. From early to late stages there is an increase in miR-17-92 levels that diminishes p38α, allowing further activation of Wnt signaling. Lgr6+ cells are then selected, as they have a proliferative advantage over the cells lacking this R-spondin receptor. Similar to other related R-spondins (e.g. Lgr5), Lgr6 is also considered a marker for adult stem cells and a potential marker for cancer stem cells (27). We now show that Lgr6+ cells from different stages of adenocarcinoma have higher tumorigenic and self-renewal potential than Lgr6- cells. Lgr6+ cells from early stages still maintain their capacity to differentiate into bronchioalveolar lineages, but this functionality is lost in later stages. This is likely due to enhanced Wnt signaling, that promotes self-renewal, and deficient p38α activity, that controls differentiation of lung stem cells. As a consequence, Lgr6+ accumulate in later stages, retaining cancer stem cell features, with active self-renewal and poor differentiation, both signatures of advanced stages in cancer.

We have therefore found a co-adjuvant mechanism, activated through human lung adenocarcinoma, which allows the selection of cells with characteristics of cancer stem
cells, higher potential to form new tumors, capacity to establish in other tissues, and rapid proliferation.

In this paper, we are contributing to the elucidation of mechanisms that promote malignancy throughout lung adenocarcinoma development. A co-adjuvant process, combined with the side-effect of oncogenic mutations, allows the selection of a population of cells with features of cancer stem cells, providing more malignant properties and advancing tumor progression. These findings add new potential cellular and molecular targets for detection, prognosis and therapies to tackle this devastating disease.

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Guinot et al.,


Guinot et al.,


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Table 1. DNA sequence of the primers used for qPCR

FIGURE LEGENDS

Figure 1. Increased Lgr6+ cells during human lung adenocarcinoma progression.

(a) Histopathology images of normal lung (left) and different stages of lung adenocarcinoma (TTF1 positive tumors). Lgr6 expression is higher in later stages.

Representative images of 15 Stage 1, 30 Stage 2 and 8 metastatic lung adenocarcinomas. (b) Detection by western blotting of Lgr6 protein expression.
correlates with imaging at different adenocarcinoma stages. Numbers are the mean of three independent experiments ± SD. Significant differences are marked with * (p≤0.05).

(c) Flow cytometry analysis of control, Stage1, Stage 2 and metastatic lung adenocarcinoma tumors for Lgr6 expressing cells. Representative experiment of 3 biological replicates. FSC stands for forward scatter and SSC stands for side scatter. (d) Immunohistology images show expression of the proliferation marker Ki67. The graph shows the quantification of Ki67 positive cells at different stages. N= 5 Stage 1, 10 Stage 2, 5 metastatic tumors (5 slides per sample). Percentage of positive cells ± standard error of the mean (S.E.M) in randomly selected fields. (e) Flow cytometry analysis of Lgr6 and Ki67 positive cells. The plots are representative of 5 Stage 2 lung adenocarcinoma tumor biological replicates.

Figure 2. Lgr6+ cell accumulation correlates with dysregulation of the miR-17-92/p38α axis during adenocarcinoma development. (a) Graph shows qPCR analysis of miR-17-92 members’ relative expression at different stages of lung adenocarcinoma compared to normal lung. Bars depict the mean, ± SD, of three independent experiments with 5 biological replicates per stage. (b) Immunohistochemical staining of early and late stages of lung adenocarcinoma, showing nuclear expression of total p53. Representative images of three biological replicates. Lower panel shows mRNA levels of p21, a direct target of p53, used to test p53 activity. (c) Western-blot shows the expression of p38α in healthy and tumor tissue from patients at different stages of lung adenocarcinoma, with metastatic referring to an invasive primary tumor. The numbers represent the relative expression in each tumor compared to its healthy counterpart tissue. One representative experiment out of 5 is depicted. (d) Expression of p38α in normal Lgr6+ lung cells, with or without overexpression of the miR-17-92 member
Guinot et al.,

miR-19. The graph shows the relative levels of p38α ± standard error of the mean (S.E.M) (N=5). (e) Protein expression of Lgr6 and p38α in normal lung or Lgr6+ or Lgr6- cells from Stage 2 lung adenocarcinoma. The numbers are relative to the lung control and normalized with tubulin levels. One representative experiment of 3 biological replicates is presented. (f) Graph shows qPCR normalized numbers representing miR-17-92 family member expression in FACS sorted Lgr6+ compared to Lgr6- cells from early (Stage 1) or later stages (Stage 2) of lung adenocarcinoma. Results are the mean, ±SD, of three independent experiments with 5 biological replicates per stage. Significant differences are marked with * (p≤0.05).

Figure 3. p38α reduction mediates Wnt signaling enhancement during adenocarcinoma progression and selection of Lgr6+ tumor cells. (a) Western-blot showing activation of Wnt canonical pathway (P-GSK3) during adenocarcinoma development. Representative image of three independent replicates. (b) Immunohistochemical analysis of canonical Wnt activation at early and late stages of lung adenocarcinoma. Representative images of 5 biological replicates per stage. (c) Graphs show mRNA relative levels of Wnt components, targets and repressors in normal lung (blank bars), early (dark) or late (grey) stages of adenocarcinoma. Results are the mean, ±SD, of three independent experiments with 5 biological replicates per stage. Significant differences are marked with * (p≤0.05). (d) Relative mRNA expression in Lgr6- and Lgr6+ cells from stage 2 tumors. Results are the mean, ±SD, of 5 tumor samples in triplicates. Significant differences are marked with * (p≤0.05). (e) Western-blot showing P-GSK3 levels in normal lung Lgr6+ cells or cells lacking p38α (p38KD), or cells combining lack of p38α and miR-17-92 downregulation (p38KD+shmiR). Representative image of three independent replicates. (f) Relative
mRNA expression of Wnt components in Lgr6+ or Lgr6+ cells lacking p38α (p38KD).
(g) mRNA expression by qPCR of Wnt targets in Lgr6+ or Lgr6+/p38KD cells. (h) Overexpression of p38α in Lgr6+ cells deficient in p38α restores pGSK3 levels. (i) p38α rescue in Lgr6+/p38KD cells restores Wnt components and Wnt targets mRNA expression. All results are the mean of 3 to 5 experiments, ±SD.

Figure 4. Stage 2 Lgr6+ tumor cells are more tumorigenic than Lgr6- cells and retain self-renewal and differentiation potential. (a) Cells from tumor masses at different stages of lung adenocarcinoma were sorted into Lgr6+ and Lgr6- populations and analysed for mRNA expression of Lgr6, p38α and lung specific markers. Results are the mean, ±SD, of 3 tumor samples in triplicates. (b) Tumor cells from Stage 2 adenocarcinoma were sorted for Lgr6+ and Lgr6- populations and seeded in soft agar to test for tumorigenic potential. Colonies that formed were stained with crystal violet. (c) Lgr6+ and Lgr6- cells from Stage 2 adenocarcinomas were injected (10⁴ cells) intravenously in SCID mice (n= 4). Only Lgr6+ cells produced tumors. The images show H&E, TTF1 immunohistochemistry, and staining of paraffin sections with a human mitochondrial antibody (green) and TTF1 (red). Representative images of three biological replicates. (d) IV injected Lgr6+ cells were detected in the lungs of SCID mice and stained with human specific mitochondrial antibody and Lgr6 or lung specific markers (SP-C, CC-10). Representative images of three biological replicates.

Figure 5. Lgr6+ adenocarcinoma cells retain tumorigenic potential and loss of differentiation capability due to increased Wnt signaling during cancer development. (a) Lgr6+ and Lgr6- tumor cells from different stages of lung adenocarcinoma were injected (10³-10⁴ cells) in parallel flanks of SCID mice (n= 10).
Guinot et al.,

Histological analysis show a tumor formed from Stage 2 Lgr6+ cells. (b)

Immunofluorescence analysis of the tumors with TTF1 and human mitochondrial antibodies confirmed their human adenocarcinoma origin. (c) Histological and immunofluorescence images of tumors from early, medium and late adenocarcinoma stages. The tissues were stained with antibodies to detect human (hMito), epithelial (E-Cad), stem (Lgr6) and lung specific (SP-C, CC-10) markers. Representative images of 8 biological replicates.
Fig. 1

(a) H&E, TTF1, and Lgr6 staining showing lung tissues from patients with Stage 1, Stage 2, and metastatic disease.

(b) Western blot analysis of Lgr6 and Tubulin expression levels in lung tissue from patients with Stage 1, Stage 2, and metastatic disease.

(c) Flow cytometry analysis of Lgr6 expression in cells from patients with Neg. Control, Stage 1, Stage 2, and metastatic disease.

(d) Ki67 staining showing lung tissues from patients with Stage 1, Stage 2, and metastatic disease.

(e) Flow cytometry analysis of Lgr6 and Ki67 expression in tumor cells and Neg. Control.
Fig. 3

a) Stage 1, Stage 2, Metastatic

Tubulin

pGSK3

1.0  2.1  5.2

b) P-GSK3  β-Catenin

Lung

Stage 1

Stage 2

Metastatic

c) mRNA relative levels to HPRT

Control  Early stage  Late stage

Lrp6  Lef1  Tcf4  Axin2  Dkk1  Gremlin  Dab2  Wif1

*  *


d) mRNA relative levels to HPRT

Lgr6- cells  Lgr6+ cells

Lrp6  Lef1  Axin2  Tcf4  Wif1  Gremlin  Dkk1  Dab2

*  *


e) Lgr6+  Lgr6+ p38KD  Lgr6+ shmiR

P-GSK3  Tubulin

f) Relative expression levels to GAPDH

Lgr6+  Lgr6+/p38KD

LeF1  Dab2  Axin2  Tcf4

*  *


g) Relative expression levels to GAPDH

Lgr6+  Lgr6+/p38KD

LeF1  Dab2  Axin2  Tcf4

*  *

h) Control  p38KD  p38α rescue

α-Tub  p38α  P-GSK3

0.1  1  0.4

i) Relative expression levels to GAPDH

Control  p38KD  p38α rescue

Lrp6  Lef1  Axin2  Tcf4  Dab2  Dkk1  Wif1  Gremlin

*  *
Fig. 4

(a) mRNA relative levels to HPRT for Lgr6- and Lgr6+ conditions.

(b) Light microscopy images of Lgr6- and Lgr6+ samples.

(c) Immunohistochemistry images showing TTF1 and hMitoc localization.

(d) Confocal microscopy images of Lgr6, SP-C, and CC-10 with hMitoc and DAPI.
miR-17-92/p38α dysregulation enhances Wnt signal and selects Lgr6+ cancer stem cell like cells during human lung adenocarcinoma progression

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