Alexandre Miguel Barata Dias

Licenciado em Bioquímica

Characterization of MEX3A RNA-binding protein in mouse stomach

Dissertação para obtenção do Grau de Mestre em

Genética Molecular e Biomedicina

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“Science is a way of thinking much more than it is a body of knowledge” – Carl Sagan.

Preface

Since a long time ago, humans are interested in discovering how live organisms function, the questions that raise more curiosity, are the ones about ourselves. Science started with rudimentary methods, just discovering our bodies anatomy, big structures as organs and how was everything related, beginning just with imagination and curiosity.

With the technology development, it was possible to start observing living beings in a smaller scale, until there past unnoticed, and consequently it became necessary to develop technology to understand what was being seen.

Considering scientific research evolution as a function, I believe humans find themselves in the exponential part of science evolution. Due to the demand for curiosity satisfaction, the current technology is more advanced than ever and, nowadays, its possible to investigate viscerally in all areas. The answers to these questions raised more questions that lead to more scientific research, becoming a cycle inherent to human condition, responsible for knowledge acquisition, society development and amelioration of human race. The consequences of human curiosity are more evident nowadays, as we find ourselves at a point in history where understanding of our surroundings and its complexity has never been so superb.

Acknowledgments

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À minha família toda, em especial à minha mãe, por todo o esforço, apoio incondicional, carinho e ter dado tudo o que pôde e não pôde durante toda a vida, principalmente na altura da licenciatura e mestrado, que sem eles nada disto se tinha concretizado e nem eu seria nada. Ao meu pai pelo apoio, pelos muito bons momentos e carinho, principalmente este ano, que sem ele não teria sido possível estudar no Porto. À minha tia Teresa, por todo o amor, carinho, apoio incondicional e felicidade que me deu ao longo deste ano. À minha avó por sempre me ter apoiado e dado tudo o que pôde. E por fim, quero agradecer ao meu irmão, o meu melhor amigo, por tudo, cuja enumeração é impossível.

Resumo

A proteína de ligação ao RNA MEX3A, está associada à diferenciação de células estaminais no intestino de rato, enquanto que no estômago, a sua função encontra-se por elucidar. É co-expressa com a proteína LGR5, em uma subpopulação de células estaminais caraterizada pela sua divisão lenta, que se encontra em criptas intestinais de camundongos. A proteína LGR5, marca diferentes tipos de células estaminais, tanto no antro como no corpo, essenciais para a recuperação da homeostase ou de uma lesão.

Aqui é relatado uma relação entre a MEX3A e a regulação de células estaminais gástricas, através da sinalização da via Wnt/β-catenin. Os camundongos *Mex3a*$^{-/-}$, possuem uma manifestação fenotipípica no epitélio gástrico. As manifestações mais acentuadas, são uma redução da espessura da mucosa gástrica e no número de células Ki67^{+}. Organóides derivados de um ratinho *Mex3a*$^{-/-}$, apresentaram uma taxa de crescimento mais lenta e dimensões reduzidas, em comparação com organóides derivados do um ratinho *Mex3a*$^{+/-}$ irmão. Recorrendo a organóides derivados de um ratinho *Lgr5*$^{+/EGFP}$, observou-se expressão de LGR5-EGFP, sugerindo a presença de células estaminais. Quando células de cancro gástrico, cresceram em meio suplementado com fatores de crescimentos agonistas da via Wnt/β-catenina, a expressão de MEX3A aumentou nas linhas celulares MKN45, MKN28, NCI-N87 e SNU-638. O marcador de células estaminais SOX2, foi utilizado para avaliar o caráter estaminal da generalidade das células cancerígenas. Nas linhas celulares MKN45 e AGS, a expressão de SOX2 aumentou quando as células cresceram em meio suplementado.

Devido às manifestações fenotípicas dos ratinhos *Mex3a*$^{-/-}$, à taxa de crescimento reduzida dos organóides derivados do ratinho knockout e à sobre-expressão de MEX3A nas células cancerígenas, aparenta existir uma relação entre a MEX3A e a regulação das células estaminais gástricas através da via Wnt/β-catenin.

Termos-chave: Estômago, Organóides, Células estaminais, Wnt/β-Catenina, MEX3A

Abstract

The MEX3A RNA-binding protein, is associated with stem cell differentiation in mouse intestine, whereas in stomach, MEX3A function is still unknown. It is co-expressed with LGR5 in a sub-population of slowly-dividing stem cells in mice intestinal crypts. LGR5 protein, marks different types of stem cells, both in antrum and corpus essential for homeostasis or injury recuperation.

Here it is reported a link between MEX3A and gastric stem cells regulation through the Wnt/β-catenin pathway signaling. *Mex3a*$^{-/-}$ mice, exhibit a phenotypic manifestation in gastric epithelium, namely a reduction of the gastric mucosa thickness and the number of Ki67^{+} cells. Organoids derived from *Mex3a*$^{-/-}$ mouse, have a slower growth rate and reduced dimensions than the organoids derived from the *Mex3a*$^{+/-}$sibling. Using organoids derived from *Lgr5*$^{+/EGFP}$ mouse, it was observed a LGR5-EGFP expression, suggesting the presence of stem cells. When gastric cancer cells were grown in medium supplemented with Wnt/β-catenin pathway agonist growth factors, MEX3A expression increased in MKN45, MKN28, NCI-N87 and SNU-638 cell lines. The stem cell marker SOX2 was used to evaluate the general stem character of the cancer cells pool. In 4 of the 5 cellular lines studied, MEX3A expression increased when cells were grown with supplemented medium. In MKN45 and AGS cell lines, SOX2 increased when cells were grown in supplemented medium.

Together, these studies suggest a relation between MEX3A and gastric stem cells regulation through the Wnt/β-catenin pathway, because of the phenotypic manifestations in *Mex3a*$^{-/-}$ mice, the slower growth rate of *Mex3a*$^{-/-}$ derived organoids and the MEX3A overexpression when cells are grown in a medium supplemented with an activator and agonist of the Wnt/β-catenin pathway.

Keywords: Stomach, Organoids, Stem cell, Wnt/β-Catenin, MEX3A

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MEX – Muscle Excess;

PAS –Periodate-schiff;

ECL – Enterochromatin-like;

GIF1 – Gastric Intrisc Factor 1;

LGR – Leucine-Rich repeat containing G protein coupled receptor;

ZNFR3 – Zinc and Ring Finger 3;

RNF43 – RING Finger protein 43;

SRY2 - Sex Determining Region Y box 2;

CCkbr – Cholecystokinin B receptor;

Vil1 – Villin1;

IFNγ – pro-inflammatory cytokine interferon gamma;

CDX1, 2 and 4 – Caudal Type homeobox1, 2 and 4;

PDX1 – Pancreatic and Duodenal homeobox1;

FGF4 – Fibroblast Growth Factor 4;

FGF10 - Fibroblast Growth Factor 10;

BMP – Bone Morphogenetic Protein;

SHH – Sonic Hedghog;

EGF- Epidermal Growth Factor;

GSK3- Glycogen Synthase Kinase 3;

APC- Adenomatous Polyposis Coli;

FDA- Food and Drug Administration;

RSPO - Roof Plate-Specific Spondin;

TFF2 – Trefoil Factor 2;

MUC5AC – Mucin5ac;

MUC6 – Mucin6;

CHGRA – Chromogranin A;

TNFRS19- TNF Receptor Superfamily Member 19;

RBP – RNA-Binding Protein;

RNP – Ribonucleoprotein;

RBD – RNA-Binding Domain;

ARM – Arginine-Rich Motif;

PUF – Pumilio/FBF;

KH – K-Homology;

NOG – Noggin.

1. Introduction

1.1. Murine and Human stomach

1.1.1. Differences between human and murine stomach

The stomach is part of the gastrointestinal tract with the main function of secreting digestive enzymes and gastric acid, assisting the digestion and preparation of the ingested material to be absorbed in the intestine. It has a similar function in the majority of mammalians.

The mice stomachs, are divided in three parts, the forestomach, a non-glandular part, the corpus or fundus and the pyloric antrum both composed by glands. Forestomach is a thin, elastic and grey colored tissue, which represents approximately two thirds of the total volume of the stomach. It is composed by a stratified squamous and keratinized epithelium. It stores the food supply, and depending on the energy requirement, releases food for progressive digestion and absorption. The forestomach allows mice to maintain a continuously state of digestion regarding the energy demand (Lee et al., 1982; Gärtner, 2001). In humans the forestomach is absent, and in contrast there is a region called proximal peri-oesophageal cardia, located in the transition zone of esophagus to corpus (Choi et al., 2014). The other third of the stomach is the glandular portion, and both parts, corpus and antrum, exhibit an inner coating of secretory active epithelium composed by small units, named glands (Lee et al., 1982).

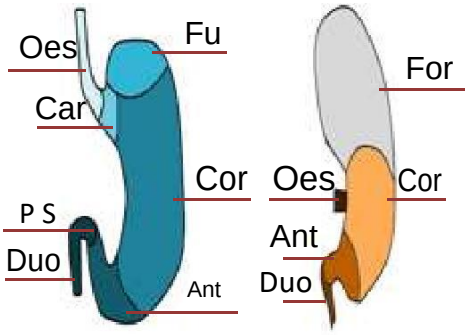


Figure 1. 1 Representation of the different anatomical parts of the human (left) and mouse (right) stomachs. Oes - Oeshopagus; Fu – *Fundus*; For – Forestomach; Car – Cardia; Cor – *Corpus*; Ant – *Antrum*; Duo – Duodenum; PS – Pyloric Sphincter;

The corpus or zymogenic glands are morphologically and cellularly different from antral or *pyloric* glands. The main function of zymogenic glands is the production of gastric acid and the release of digestive enzymes. The antral glands help the transition of digested food to the intestine by entrance in the duodenum (the switch-over part between stomach and intestine), through mucous secretion and

production of hormones (Scanlon and Sanders, 2015). The differences between both glands in mouse beyond the different dimensions are the presence of different cellular lineages.

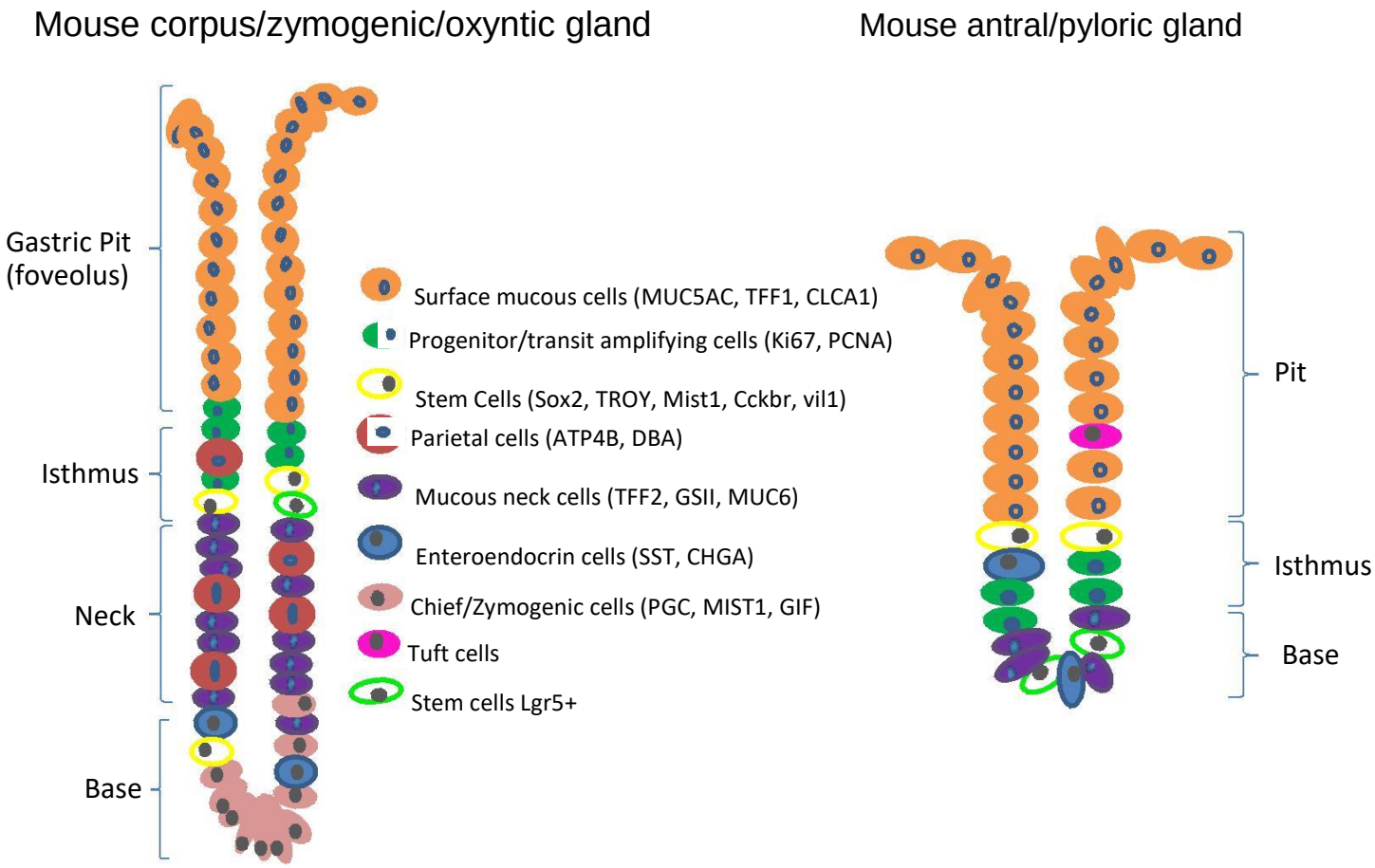


Figure 1. 2 Schematic representation of corpus and antral glands with their cellular lineages. Corpus glands are larger in length and fission size. In parentheses are described different molecular markers for the same lineage except to stem cells that each marker label different stem cells. The left and right bars depict the different regions of the glands

The transition zone of *antrum* to *corpus* is composed by glands that exhibit mixed characteristics from both glands designated mucoparietal (Demitrack et al., 2015; Lee et al., 1982).

1.1.2. Mouse cellular lineages and respective molecular markers

1.1.2.1. Pit and neck mucous cells

Gastric glands harbour several cellular lineages with distinct functions. In homeostasis, these cells are driven from tissue resident stem cells (Mills and Shivdasani, 2011; Leushacke et al., 2013; Arnold et al., 2011). Adult stem cells ensure the gastric epithelial turnover within a 7-10 day rate (Barker et al., 2010a).

In the distal part of the glands, in the region called pit, are found mostly, the pit mucous cells, characterized by mucous production. Pit cells have a life span of approximately 3 days (Karam and Leblond, 1993a). The most accepted biomarker for this cellular lineage is the glycoprotein mucin5ac. There are also mucous producing cells in the neck region, these ones are distinguished by the expression of mucin6 (Schlaermann et al., 2016). Mucous is characterized by the presence of several mucins, the periodate-Schiff (PAS) reaction can be used to stain these mucins and therefore mark pit and neck mucous cells (Thornton et al., 1989).

1.1.2.2. Proliferative cells

The isthmus is characterized by the abundance of proliferative cells. From this region occurs a bidirectional migration of progenitor cells towards the pit or the base of the glands (Ramsey et al., 2007; Lee et al., 1982). These cells possess a turnover time of 3 days or less (Karam and Leblond, 1993b). A common molecular marker used in proliferative cells identification is the KI67 protein, which is associated with ribosomal RNA transcription and cellular proliferation (Bullwinkel et al., 2006).

1.1.2.3. Neuroendocrine cells

Endocrine cells are found along the gastrointestinal tract, which are responsible by regulation of other cells function, through hormones and peptides secretion (Rehfeld, 1998). Endocrine cells are located mostly in the gland base, and exhibit a turn-over time of approximately 3 months (Karam and Leblond, 1993c). At least six different types of endocrine cells were identified in the gastric mucosa. The following endocrine cells are the well-described, enterochromatin-like cells (ECL cells) that secrete histamine, Delta cells also called D-cells distinct by somatostatin secretion, gastrin-secreting cells or G-cells responsible by the gastrin hormone production, A-cells that synthesize glucagon, enterochromaffin cells which secret serotonin and X-cells also known as A-like cells which can be distinguished from the others by Ghrelin production (Grube and Forssmann, 1979; Cockburn et al., 2013; Enrico et al., 1975). Since chromograninA expression is restricted to endocrine cells, this protein, among others, is widely used as molecular marker to identification of these cell lineage (Oberg, 1998).

1.1.2.4. Parietal cells

Parietal cells are found dispersed through the neck and base regions of corpus glands. These cells are responsible for hydrochloric acid production and secretion, through a coordinate action of endocrine, paracrine and neuroendocrine pathways (Mills and Shivdasani, 2011; Rehfeld, 1998).

These cells are the ones with the larger dimensions found in the stomach and have a low turnover rate of approximately 54 days. They are originated in the isthmus, and migrate bidirectional inward to the neck and eventually to the base, or outward to the pit (Karam, 1993). Parietal cells exhibit an unique intracellular structure denominated *Canaliculus*, that has the function to increase the cell superficial area, assisting in the secretions. In their membrane there is a characteristic enzyme denominated hydrogen potassium ATPase (H+/K+ ATPase), responsible for the production of H^+ ions concentration characteristically of gastric juice, approximately 3 million times superior than the concentration found in blood (Martinsen et al., 2005). Due to this unusual characteristic, H+/K+ ATPase is the most used molecular marker for parietal cells identification.

1.1.2.5. Chief or zymogenic cells

These cells occupy the corpus glands base, their main function is the secretion of proteolytic enzymes such as pepsinogen, that assist in the organic material degradation (Jun and Shinichiro, 1998). Chief cells possess the slowest turnover of all gastric cell lineages, being estimated in order of half of a year (Karam and Leblond, 1993a). These cell are the only ones derived from neck cells, passing through a pre-zymogenic stage before maturation, whereas the other lineages derive directly from isthmal cells (Karam and Leblond, 1993c). For chief cells immunostaining, there are at least two molecular markers used, the transcription factor Mist 1, associated with proteins secretion, and the Gastric Intrisic Factor 1 (GIF1) a glycoprotein necessary to B12 vitamin absorption (Lennerz et al., 2010), (Howard et al., 1996), (Alpers and Russell-jones, 2013).

1.1.2.6. Gastric stem cells

Adult stem cells reside within organs and tissues, fueling their growth and maintaining their regeneration throughout adult life. These cells exhibit multipotency, possessing the ability to produce one or more cellular lineages. In adult homeostasis, stem cell fate decisions are controlled by extrinsic signals of their microenvironment or niche (Watt et al., 2000). These cells are located in the isthmus and base of corpus glands, or in the base of antral glands (Barker et al., 2010a).

The turnover time varies depending on the stem cell type. Adult stem cells molecular markers are not well-characterized. The best well-characterized biomarker of adult, quiescent and homeostatic, stem cells, in several tissues, including the antrum and the corpus, is the membrane protein Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5). This membrane receptor belongs to the leucine

rich repeat containing G protein-coupled receptor (LGR) family. Until recently it was thought to be present only in stem cells in the base of antral glands (Barker et al., 2010a),. More recently Leushhacke et al. demonstrated that it also exists in the region of the isthmus in the body (Leushacke et al., 2017). When activated by molecules of the Roof Plate-Specific Spondin (Rspondin) family, the complex LGR-Rspo inactivates an E3 ubiquitin ligase zinc and ring finger 3 (ZNRF3) and ring finger protein 43 (RNF43), which are Wnt/β-catenin pathway antagonists, activating the pathway activity (Kretzschmar and Clevers, 2017; Kazanskaya et al., 2004).

It has been proven that a specific cell type that expresses the transcription factor Sex Determining Region Y box 2 (SRY2 also known as SOX2), label a different and more rare population of cells than LGR5. These cells have the potency to originate whole monoclonal glands (Arnold et al., 2011). Mist1, is also a transcription factor, that marks a type of quiescent stem cells in the gastric corpus isthmus (Hayakawa et al., 2016). Both SOX2 and Mist1 do not label exclusively stem cells (Stange et al., 2013). Hayakwa et al., demonstrated that the cholecystokinin B receptor (Cckbr or CCK2R) protein also identifies a type of quiescent stem cells that are able to transform in $LGR5^+$ stem cells (Hayakawa et al., 2014). Qiao et al. shown that a subpopulation of reserve stem cells, localized in the neck and base of antral glands, is marked by the expression of Villin (Vil1). These cells are more rare than $LGR5^+$ cells, divide slowly and form monoclonal glands only when mice were treated with pro-inflammatory cytokine interferon-gamma (IFNγ), suggesting these cells do not contribute to epithelium homeostasis (Qiao et al., 2007). More studies will be needed to definitely establish whether $Villin^+$, $LGR5^+$, $SOX2^+$, $Cckbr^+$ and $mist1^+$ cells are different types of stem cells that act independently to maintain the stomach homeostasis, or whether these cells are hierarchically related to each other.

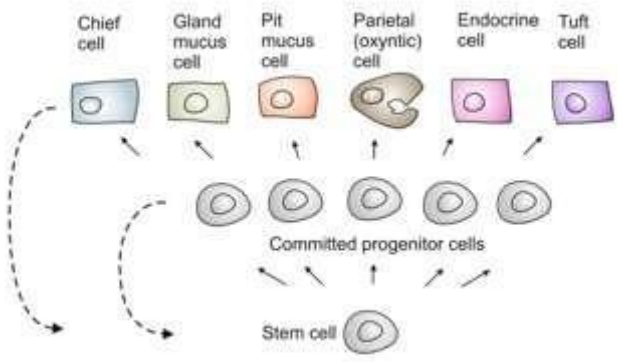


Figure 1. 3 Schematic representation of stem cells differentiation in gastric cellular lineages. Stem cells divide to give rise committed progenitor cells which in turn can proliferate and differentiate into the respective cell type of the gastric gland. Differentiated cells can gain stem cell capacity indicated by dashed arrow (Pompaiah and Bartfeld, 2017).

1.1.3. Molecular pathways of murine stomach embryogenesis

Although it is not known precisely how gastric development occurs, several molecular mechanisms and signaling pathways associated with differentiation, patterning and growth of the gastrointestinal tract have been elucidated (Lewis and Tam, 2006; Jensen et al., 2000; Kolterud et al., 2009). There is no exact model that explains how all steps occur, but there are small templates that put together some pieces of the puzzle.

The formation and patterning of the gut is an ancestral process, which probably dates back to the first organisms of the animal kingdom to exist. These processes, have probably, been controlled by genes with similar functions in the last 500 million years. In mice, the gastrointestinal tract develops from the embryonic gut. The epithelium, is derived from endoderm, and mesenchyme is originated through the migration and condensation of mesoderm around endoderm (Wells and Melton, 1999; Ramalho-santos et al., 2000).

There are several well-characterized transcription factors associated with control and regulation of mouse embryogenesis, such as Caudal type homeobox 1, 2 and 4 (CDX1,2 and 4), pancreatic and duodenal homeobox 1 (Pdx1), Fibroblast Growth Factor 4 (FGF4), hox proteins and β-Catenin (Wells and Melton, 1999), (Lewis and Tam, 2006).

During embryonic development, inhibition of the Wnt/β-Catenin pathway occurs, due to the expression of the transcription factor BARX Homeobox 1 (negative regulator of secreted frizzled-related protein 1 and 2) in the mesenchyme, promoting a gastric specification of the epithelium rather than intestinal (B. Kim et al., 2005), (Rattner et al., 1997), (Verzi and Shivdasani, 2008).

McCraken et al. have shown that unlike the intestine, the Wnt/β-Catenin and Fbroblast Growth Factor 10 (FGF10) pathways act epistatically with the Bone Morphogenetic Protein (BMP) pathway, but independently, to conduct morphogenesis of gut tube structures. The retinoic acid pathway, is required for the differentiation of the gut tube into a posterior foregut fate, namely the antrum (McCracken et al., 2014).

Hedgehog pathway is also related to cell fate decisions and patterning of the stomach (Kolterud et al., 2009). This pathway is composed by a set of genes such as Sonic hedgehog (Shh), Indian hedgehog and Desert hedgehog. Santos R. M. et al. demonstrated that mutants for Shh, do not develop a functional gastric epithelium, and exhibit a partial transformation into intestinal epithelium (Ramalho-Santos et al., 2000).

1.1.4. Molecular pathways of murine stomach homeostasis

After the decision to specify the endoderm in gastric epithelium occurs, the proliferation and differentiation of several cell lineages begins. These processes are dependent on time-related mesenchyme-epithelium interactions, and several pathways in common with embryonic retain development (Wells and Melton, 1999).

In the adulthood, homeostasis of the stomach is dependent on tissue-resident stem cells. Some of the signaling pathways that ensure stomach homeostasis are the Wnt/β-Catenin pathway (Radulescu et al., 2012; Barker et al., 2010a), Notch (Demitrack and Samuelson, 2016; Demitrack et al., 2015), Epidermal Growth Factor (Fukuda and Yasugi 2005), FGF10 (Nyeng et al., 2007), gastrin (Jain and Samuelson, 2006), BMP (Bartfeld and Koo, 2017) and Hedgehog (Ramalho-santos et al., 2000; Lees et al., 2005).

In the adult corpus epithelium, Shh is secreted by the parietal cells, and regulates gastrin production by the endocrine G cells. When the Shh produced in the parietal cells is inhibited, hyperproliferation of the foveolar epithelium occurs (Brink et al., 2001), (Konstantinou et al., 2016). The BMP pathway is associated with the stimulation of stem cell differentiation, and when inhibited, hyperproliferation and poor compartmentalization occurs (Kaestner et al., 1997). In intestine, a BMP gradient is established with low expression in the crypts and high expression in the *villi*, where the cellular differentiation is higher, contributing to restrict the stem cells to the crypts. This gradient is achieved due to the activity of Shh that is highly concentrated in the mesenchyme that surrounds *villi* (Shyer et al., 2015). In the stomach, the expression pattern of bmp4 differs from that of the intestine, because Shh is secreted only by parietal cells in the neck region, leading to a high concentration of Bmp4 in this region, where stem cells are scarcer (Brink et al., 2001). This suggests that Shh secretion by parietal cells shapes the negative area for stem cells microenvironment, which can be established in the isthmus and base of the gland where parietal cells are scarcer. Shh pathway also regulates Notch and FGF10 pathways (Bartfeld and Koo, 2017).

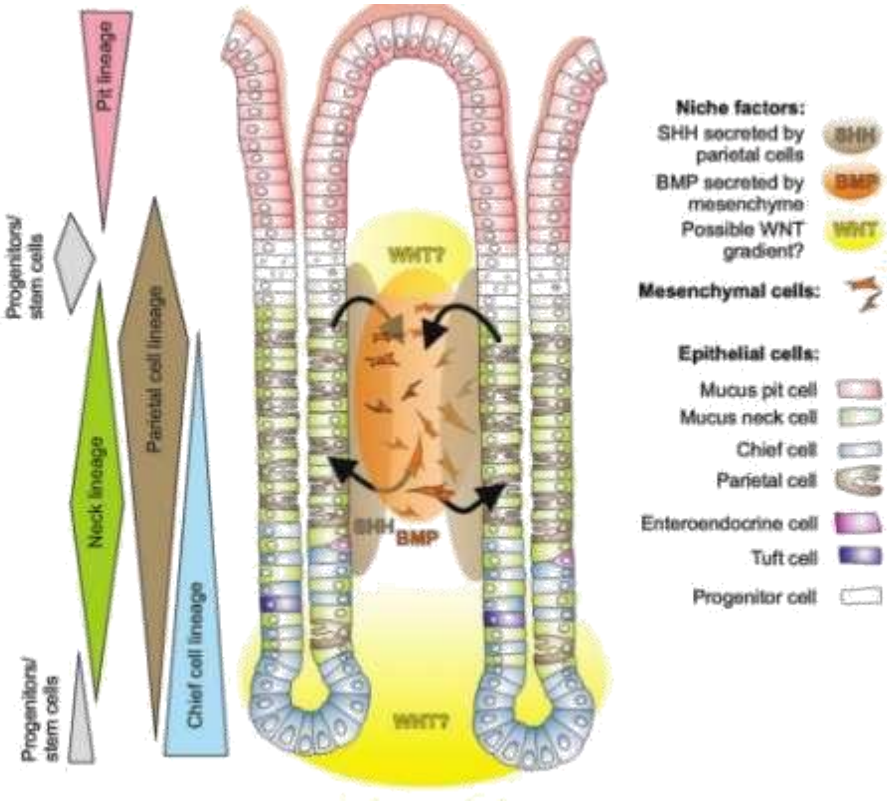


Figure 1. 4 Distribution of cells types and niche pathways in the gastric corpus gland. On the left side are represented the abundance of each cell lineage. The circles between the two glands represent gradients of niche pathways activity. The Shh high concentration in the neck region leads to the mesenchymal BMP expression. There is only indirect data for the proposed WNT gradients represented here (Bartfeld and Koo, 2017).

A similar phenotype is achieved when overexpression of the transforming growth factor-α (an EGFR ligand) (TGF-α) is induced, leading also to hyperproliferation of the foveolar epithelium and loss of parietal cells (Goldenring et al., 1996; Sharp et al., 1995).

Notch signaling is restricted to the isthmus, and is essential to the maintenance of the stem cell compartment. Overactivation of Notch, causes cellular hyperproliferation, generating corpus adenomas and antral polyps possibly due to the downstream activity of the mammalian Target of Rapamycin (mTOR) pathway (T.-H. Kim and Shivdasani, 2011; Demitrack et al., 2015; Syu et al., 2016).

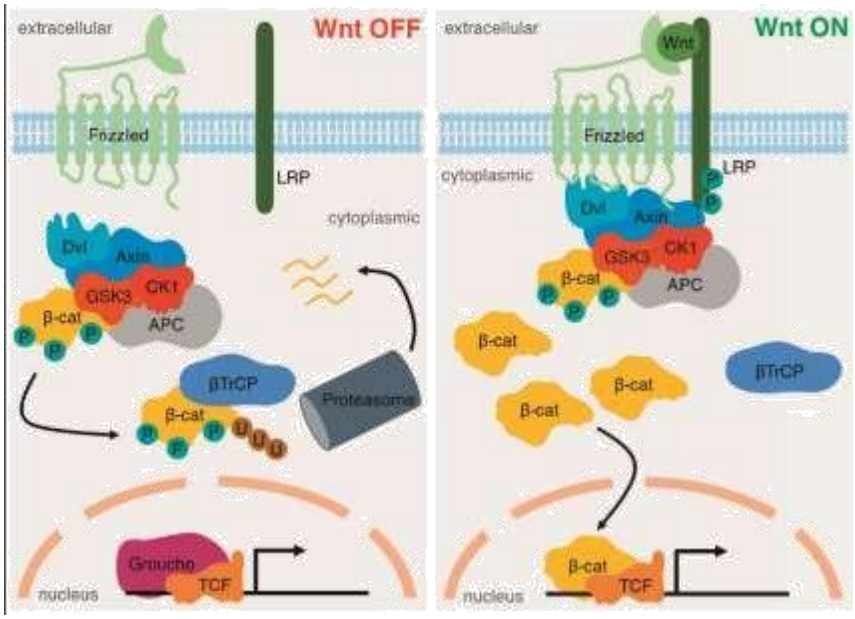


Figure 1. 5 Schematic representation of the canonical Wnt/β-Catenin pathway, which is activated by the Wnt ligands (right) and inactivated in the absence of these (Kretzschmar and Clevers, 2017).

In the intestine, the Wnt/β-Catenin pathway is the best characterized pathway and has a central importance in stem cells, whereas in the stomach, its role is not fully understood. The canonical Wnt/β-Catenin pathway is represented in the figure below.

TROY and LGR5, two adult gastric stem cell markers, are targets of the Wnt/β-Catenin pathway (Stange et al., 2013), (Barker et al., 2010a). When over-activated in stem cells via deletion of either glycogen synthase kinase 3 (GSK3) or Adenomatous Polyposis Coli (APC), the Wnt/β-Catenin pathway induces hyperproliferation and formation of polyps in the antrum and corpus (Radulescu et al., 2012).

1.2. Research study models

1.2.1. Animal models for research

Probably, due to the inexistence of a study model similar, in such a way, to the human being, its very complicated the extrapolation of the knowledge acquired of studies, to be directly applied in humans. Animal models are the ultimate barrier to overcome before human trials. Approximately 90% of new drugs tested in clinical trials fail to obtain Food and Drug Administration (FDA) approval (Gurwitz, 2001).

Animal models, although imperfect, are an irreplaceable tool in biomedical research. There are several animal models used in scientific research, such as mouse, rat, xenopus and zebrafish. For different studies, animal models may vary, for example for cell cycle studies, flies and worms are great models, but they have the limitation of a simpler physiological system than the mouse. Mice genome is just approximately 10% smaller than humans, in term of base pairs (European Bioinformatics Institute, 2017;

Mural et al., 2002). Despite this genomic resemblance, regarding to anatomy and physiology, are also similar to the human being. They are characterized by a relative short lifespan (1 mouse year equals approximately to 30 human years). Mice reproduce relatively quickly, possessing a gestation time is around ten weeks. To enlight a role of a gene, mice are a relatively simple animal to generate transgenic or knockout models. Taking these advantages, *Mus musculus* was the main animal model opted to this study (Yourgenome, 2017; Spencer, 2012).

The bigger pitfalls from mice models regarding to genetic research, is the insufficient representation of the natural genome diversity, due to usage of a single strain of inbred mice (Gurwitz, 2001).

1.2.2. Two dimension, *in vitro*, mammalian cell culture models

Due to the complexity of animal models, the study at a cellular and molecular level becomes quite difficult, requiring the use of simpler study models (Shamir and Ewald, 2014). As such, another model largely used in several areas such as neuroscience, cardiology or cancer research is the 2 dimension (2D) culture of mammalian cells (Goodspeed et al., 2016).

Briefly, this method is based on the growth of cells *in vitro*, under controlled biological, chemical and physical conditions, suspended or adhered (Carrel and Burrows, 1911). There are mammalian 2D cell culture systems, that consist in the establishment of human tumor-derived or cancer cell lines, primary cell lines and embryonic stem cell lines (Sharma et al., 2010; Martin, 1981). In general, due to their lower complexity, in scientific research these culture systems are mainly used for studies of the molecular mechanisms associated with various biological processes. Some of these processes are the ability of self-renewal of stem cells, cell differentiation and the carcinogenic process (Chowdhury et al., 2010; Takahashi and Yamanaka, 2006; Pan et al., 2011).

Although most cell culture studies have been conducted on 2D systems to date, there are several limitations inherent to this method. The standard procedure of screening compounds starts with the 2D cell culture-based tests, followed by animal model tests, to clinical trials. Until 2010, in cancer drug research, only about 10% of the new compounds tested, progressed successfully for clinical trials (Hait, 2010).

1.2.3. Three dimensional, *ex vivo,* cell culture models

In vivo, cells are surrounded in the three spatial dimensions by a totally different microenvironment than that provided by 2D cultures (Edmondson et al., 2014). Cells are in organized structural arrangements, and a specific chemical, biochemical and physical environment provided by the extracellular matrix (ECM). In mammals, almost all epithelial cells are supported by ECM (J. Lee et al., 2008). Consequently

the need emerged for the development of more complex cell culture systems that would mimic biological systems at a higher level of complexity (Shamir and Ewald, 2014). In order to bridge the gap of information extrapolated from 2D culture systems and animal models, 3D cell culture systems were created (Pampaloni et al., 2007; Haycock, 2010).

The concept of 3 dimensional (3D) cultures systems was first thought due to the pioneer work of Mina J. Bissell and her team. They were the first trying to elucidate how ECM influences the maintenance of tissue specificity, cellular shape and functions (Bissell et al., 1982). The discovery of different tissues interactions, and how they could modulate the genes expressions program of cells, consequently contributing to cell differentiation or stem cell maintenance was a crucial step for 3D systems development (Fuchs et al., 2004; Mazzoleni et al., 2009). It was shown that the microenvironment has a dramatic influence on the cellular behavior and 2D culture systems do not consider this important factor. Nowadays, it is known that cellular exposure to spatial restrictions established by a 3-dimensional scaffold defines how cells react to these signals from the surrounding microenvironment. Due to the niche, cells alter their gene expression resulting in the modulation of differentiation, proliferation, apoptosis, shape, movement and structure formation (J. Bin Kim et al., 2004; Li et al., 1987; Schmeichel and Bissell, 2003).

Briefly, 3D culture systems differ from 2D cultures in the recreation of an organic and complex scaffold, where cells are grown. In these cultures, cells are exposed to a more native microenvironment, providing different stimuli and making possible a cellular behavior more similar as the one that occurs *in vivo*, thus resulting in distinct cellular arrangements and structures formation (Vinci et al., 2012). Normally, these models are composed by porous substrates that can support differently cellular proliferation, organization, and differentiation (J. Lee et al., 2008). It is now feasible to perform studies, that address cell-ECM interactions, and enhanced studies about cell-cell interactions (Pampaloni et al., 2007; Li et al., 1987; Mazzoleni et al., 2009). There are currently more than 100 different biomaterials to be used in extracellular matrices depending on the study. It is possible to use different types of matrices, namely, biological scaffolds, polymeric hard scaffolds and micropatterned surface microplates. Biological matrices composition, commonly include, but are not limited to, fibronectin, collagen, laminin, and gelatin (Ravi et al., 2014).

It is possible to make 3D culture systems without a solid scaffold, for which there are three different models: hanging drop microplates, spheroid microplates containing Ultra-Low Attachment (ULA) coating and microfluidic 3D cell culture.

Spheroids, are the most common multicellular arrangement that allows cells to interact with each other and the ECM. Spheroids formed in 3D culture systems share some characteristics with *in vivo* structures, namely tumors, such as the existence of proliferation gradients or some degree of differentiation (Larson, 2015).

The paradigm has been changing, and the spheroids, although essential, are more related to the study of cancer. Currently there are 3D culture systems that enable the maintenance of basic characteristics of a normal adult tissue indefinitely (Barker et al., 2010a).

1.2.4. Organoid culture history

Organoids, also called “mini organs”, are a 3D culture method that allows growing the small units of animal organs, using an artificial media and resulting in self-organized structures, which mimic the natural physiological structures. The establishment of these culture systems, is only possible due to self-organizing, self-renewal and multipotency capacities of adult stem cells (Shaker and Rubin, 2012). In 2007, the intestinal stem cell marker, LGR5 (also known as Gpr49) was identified (Barker et al., 2007). This elucidation, made possible the development of an organoid culture method, for the first time, in 2009 (Sato et al., 2009). Murine intestinal $LGR5^{+}$ stem cells, were single sorted and cultured with a combination of their, previously described, natural and essential niche growth factors, Epidermal Growth Factor (EGF) an EGFR ligand, to promote cell proliferation (Dignass and Sturm, 2001); R-Spondin, a Wnt/β-Catenin pathway agonist (K.-A. Kim, 2005); Noggin, a BMP pathway antagonist (Haramis et al., 2004) and a 3D solid matrix, rich in laminin (because laminin, α1 and α2 is enriched at the crypt base) named matrigel (Sasaki et al., 2002; Sato et al., 2011; Hughes et al., 2010). Stem cell proliferation and expansion was observed, together with the formation of structures that contained differentiated cells, corresponding to all cell lineages that exist in the murine gut. Moreover, these cellular structures even self-organized into domains that resembled intestinal crypts. These organoids were long-lived, appear to posess unlimited expansion ability, could be split and re-seeded, frozen and thawed (Sato et al., 2009).

Since this landmark method was published, various organoids were grown from different tissues (X. Li et al., 2014) and animals, including humans (Petersen et al., 2014). They were grown from embryonic stem cells (David A. Turner et al., 2016), induced pluoripotent stem cells (McCracken et al., 2014) and tumours (Fujii et al., 2016; Hubert et al., 2016). Organoids were grown from several tissues such as stomach, intestine, eosophagus, liver, thyroid, optic cup, pitutary gland, inner ear, skin, pancreas, prostate, fallopian tube, gallblader, sallivary gland, lingual, lung, retina, mammary gland, kidney and brain tissues from mice and humans (Fatehullah et al., 2016; Pompaiah and Bartfeld, 2017; Huch and Koo, 2015; Cañadas and Barbie, 2017).

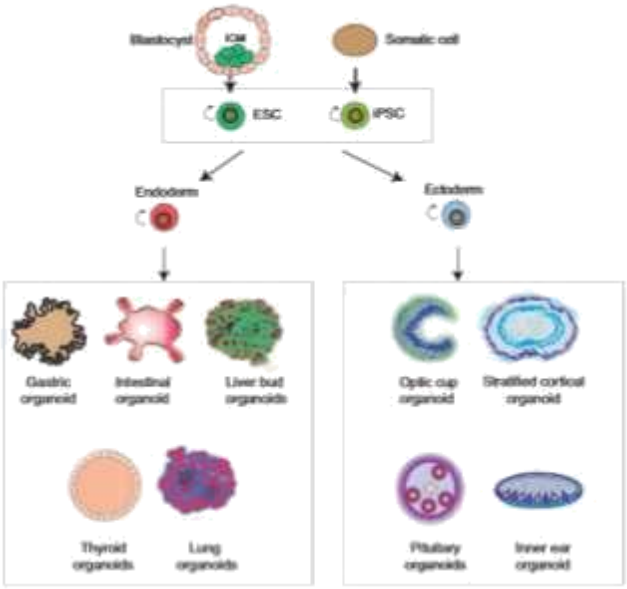


Figure 1. 6 Schematic representation of organoids derived from ESCs or IPSCs. ESCs or IPSCs can be conduct into all the germ layers. Next to this specification (endoderm, mesoderm or ectoderm), cells can generate different types of organoids according the modulation of differentiation. These organoids formation faithfully mimic the in vivo embryonic development steps (Huch and Koo, 2015).

There are several organoid applications, namely study of stem cell behavior, stem cell niche components, differentiation factors, tissue homeostasis, genetic screening, drug screening, modeling of disease and pathogen–epithelia interactions, embryonic development, lineage specification, among others (Fatehullah et al., 2016). Organoids can be grown as well, using an air-liquid interface system (Nadauld et al., 2014).

1.2.5. Gastric organoids, *ex vivo*, 3D culture systems

Due to the identification of $LGR5^+$ adult stem cells in pyloric antrum, the development of a long-lived, *in vitro*, 3D culture system of antral gastric organoids was conducted from single sorted $LGR5^+$ stem cells (Barker et al., 2010a). The first gastric culture was carried out in the same way as intestinal organoids (Sato et al., 2009), except that for the antral gastric organoids it was also necessary the addition of growth factors Wnt3a in form of conditioned media (Carmon et al., 2012; Willert et al., 2003), FGF10 for budding events (Nyeng et al., 2007) and gastrin further Rspondin, Noggin, EGF and matrigel. Gastric organoids formed cystic structures (Barker et al., 2010a).

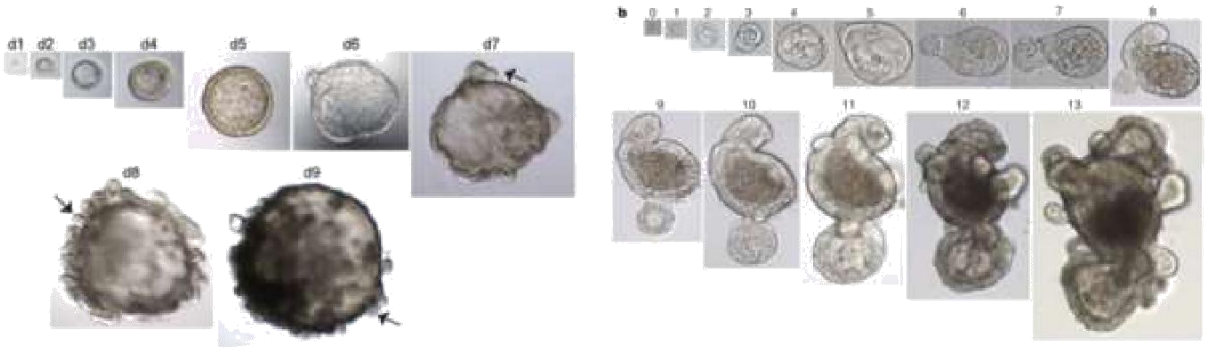


Figure 1. 7 Representative images of intestinal and antral organoids.On the left is a representative example of a unique growing antral organoid derived from a single lgr5^{+} stem cell. Arrows show the gland-like domain buds starting to form at day 5. Magnifications: days 1–4, 403 magnification; days 5–6, 203 magnification; days 7–8, 103 magnification; and day 9, 53 magnification. (Barker et al., 2010). On the right panel is a representative example of a unique growing intestinal organoid derived from a single lgr5-GFP^{hi} cell. Numbers above the images are the days of growth. magnifications: days 0–4, x40; days 5–7, x20; days 8–11, x10; days 12 and 13, x4 (Sato et al. 2009).

When cultured in normal conditions, organoids expressed various gastric epithelial markers, such as Mucin 6 (MUC6) marking mucous neck cells, pepsinogen C and gastric intrinsic factor labeling chief cells but no expression of enteroendocrine or pit cell markers was observed. To overcome this limitation, the authors reduced the Wnt3a concentration in the culture media, resulting in the formation of differentiated cells, evidenced by the expression of TFF2 in progenitor neck cells (Quante et al., 2010), scattered immature ChromograninA^{+} positive enteroendocrine cells and Mucin 5AC (MUC5AC), a typical pit mucous cell marker (Barker et al., 2010).

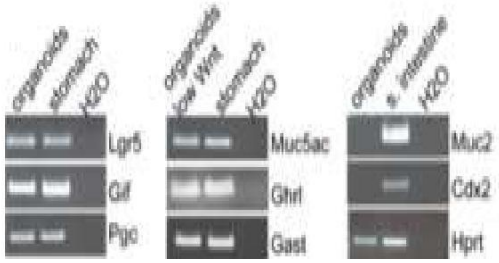


Figure 1. 8 Western blot analysis of several molecular markers of gastric and intestinal lineages. Comparison between stomach proteins and antral gastric organoids proteins. Molecular markers characteristics of small intestine lineages were used as control (Barker et al. 2010a).

In 2013, corpus organoids were grown for the first time, using the same conditions than antral organoids, from entire glands and isolated single chief cells that express TNF Receptor Superfamily Member 19 (TNFRS19 also known as TROY), and Mist1. Interestingly, TROY^{+} parietal cells did not have the ability to form long-term organoids culture. The organoids driven from these TROY^{+} stem cells, had no enteroendocrine or parietal cells markers, but expressed the other epithelial differentiation markers (Stange et al., 2013). In 2015, Schumacher et al., developed an *ex vivo* co-culture system of corpus glands with immortalized stomach mesenchymal cells (ISMCs), as described by (Zauberman et al.,

1993). The corpus glands were cultured as described by (Barker et al., 2010a), grown in an insert with pores, and transferred to a well with IMSCs plated with medium, at the bottom of the well. With this co-culture system, parietal cells were formed and maintained for several passages. Without ISMCs co-culture Schumacher et al. organoids formed parietal cells in the initial seeding, but their number decreased over culture time (Schumacher et al., 2015).

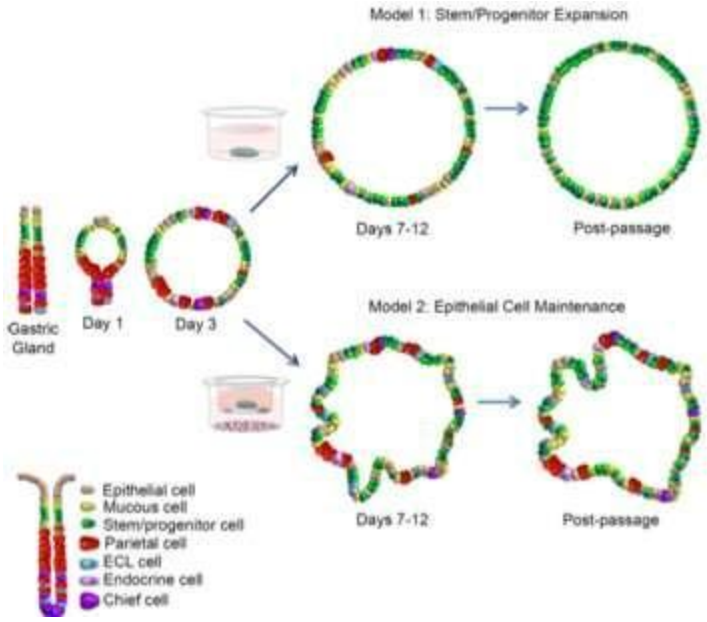


Figure 1. 9 A schematic diagram representing two distinct organoid culture methods. Model 1 (above) represents a system whereby stem/progenitor cells are expanded, the method described by Barker et al. Model 2 (below) represents a culture system of maintained epithelial cells due to a co-culture with immortalized mesenchymal cells (Schumacher et al. 2015)**.**

To grow human stomach driven organoids for the first time, it was necessary the addition of nicotinamide (an apoptosis inhibitor) to the culture medium (Avalos et al., 2005), to promote an initial organoid formation. It was also necessary the addition of a Transforming growth factor β inhibitor, to increase the culture lifespan to a maximum of half a year, by inhibition of exaggerate proliferation (Zhang, 2010). Using the same culture conditions, it was possible to form human antral organoids at least for 3 months, the corpus organoids were maintained for 1 year. These organoids expressed all differentiation markers except for parietal cells marker and Enterochromaffin-like (ECL) cells (Bartfeld et al., 2015). In 2016, Schlaermann et al. made a *quasi*-immortal gastric proliferative organoids culture, that was possible to be transformed in differentiate organoids by withdrawal of Wnt3a and Rspo1 (Schlaermann et al., 2016).

Gastric organoids derived from pluripotent stem cells (PSCs), induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) were also grown (McCracken et al., 2014). An interesting particularity, organoids grown from ASCs, in the initial seeding, contain mesenchymal as well epithelial cells. During expansion and re-plating, ASC-derived organoids become purely epithelial and PSC-derived organoids

maintain epithelium and mesenchyme, but have limited expansion capacities (Wells and Spence, 2014; Spence et al., 2011).

1.3. Genetic Regulation

1.3.1. RNA-binding proteins

DNA is the molecule that encodes genetic predispositions related with the growth, development, functioning and reproduction of all known living organisms. The temporal and spatial regulation of DNA, allows the increase or decrease of different genes expression, in distinct tissues of the same organism. It is due to the existence of genetic regulation, which is obtained, from a single genome, a functional multicellular organism. The regulation and control of genes is achieved through several mechanisms that act at the level of transcription, RNA processing and post-translational modifications. These mechanisms are inherently associated with regulatory biomolecules, in general constituted by RNA or proteins (Lewin, 2000).

One way by which genetic expression regulation can occur, is with the usage of RNA-binding proteins (RBPs) (Jansen et al., 1995). RBPs orchestrate all aspects of RNA fate and functions since its biosynthesis to an eventual degradation, becoming essential in several biological processes such as sex determination, metabolism, neurogenesis, stem-cell proliferation, erythropoiesis and imperative in embryogenesis. Defects in their normal function can be associated with a variety of human pathologies including cancer (Kuersten and Goodwin, 2003), (Gebauer and Hentze, 2004; Wurth and Gebauer, 2015; Castello et al., 2013).

When RBPs interact with RNA, ribonucleoprotein (RNP) complexes are formed, although all RBPs bind to RNA, they do that with different specificities and affinities depending on the RNA sequence. To RBPs network, achieve gene expression regulation through mRNA stability, cellular localization, function and translatability control, the presence of several regulatory elements in the mRNA chain, to assist and conduct regulation, are needed (Glisovic et al., 2008). These RNA cis-regulatory elements, are frequently located in the 5’ or 3’ untranslated regions, and can be recognized as binding sites for the trans-acting factors RBPs by specific domains (Preiss and Hentze, 2012). The three prime untranslated region (3’-UTR), is the part of the RNA sequence between the stop codon and the start of the poly(A) tail, and has a great variety of regulatory functions (Moore, 2005). There are several known sequences in this region, such as the polyadenilation signal (PAS), or the most common, the adenylate uridylate (AU-rich) elements (AREs). AREs, are known for being proteins bindings signals, and have regulatory functions by translation repression or enhancement due to mRNA destabilization or stabilization (Deshpande et al., 2009; Matoulkova et al., 2012). A basic AREs sequence, is the motif “AUUUA”, which can occur in various repetitions and lengths along the mRNA chain (Zubiaga et al.,1995).

On the other hand, RBPs possess numerous RNA binding domains (RBDs), also known as RNA recognition motif (RRM). One RBP may have multiple RBDs, and also domains that interact with other proteins (Glisovic et al., 2008). The well-described domains are the K-Homology (KH) type I and II, RGG (Arg-Gly-Gly) box, Sm domain, DEAD/DEAH box, zinc finger (mostly C-x8-X-x5-X-x3-H type) motif, dsRNA-binding domain, the Arginine-Rich Motif (ARM), cold-shock domain, Pumilio/FBF (PUF or Pum-HD) and the Piwi/Argonaute/ Zwille (PAZ) domain (Glisovic et al., 2008; Burd and Dreyfuss, 1994).

1.3.2. Mex-3 RNA binding family member A;

MEX3A RNA binding protein, is codified by *Mex*3a gene, in humans, is located in chromosome 1q22 and possesses a size of 9986pb. The citoplasmatic and nuclear protein MEX3A, has a molecular weight of 54KDa with a 520 amino acids size chain. MEX3A has three RNA interaction domains, being two of these, KH sequences, and the other a carboxy-terminal zfRING finger domain (C3HC4 type) which binds two zinc cations.

In humans, this gene belongs to a conserved across evolution family, denominated *MEX*, that its composed by four homologues genes, *ME3XA*, B, C and D (Buchet-Poyau et al., 2007). *MEX* genes orthologues, are presente in several other species, as like *Caenorhabditis elegans* and *Mus musculus* (Draper et al., 1996). Biological relevance of *MEX* family is still unknown, and is starting to be explored. In *C. elegans*, MEX-3 act as a translational repressor that regulates blastomere identity during an early embryogenesis stage (Draper et al., 1996). In adult worm, MEX-3 has been associated with germ line totipotency maintenance (Ciosk and Priess, 2006). MEX3B, was associated with innate immune response and fertility in mice (Yang et al., 2016). MEX3C, was related with the decay of HLA-A allotypes, in human embryonic kidney 293 cells (Cano et al., 2012). In human gastric cell lines, *MEX3A* expression inhibition, was related with cells proliferation capacity reduction (Jiang et al., 2012). Pereira et al., have proved that MEX3A has direct implications in epithelial intestinal cells differentiation, by CDX2 expression decrease, putatively as a translation repressor. CDX2 plays a crucial role in intestinal cell fate specification, both during normal development and in tumorigenic processes involving intestinal reprogramming, associating MEX3A with stemness maintenance (Pereira et al., 2013). Recently, Barriga et al. unveiled that MEX3A is co-expressed with LGR5 protein, in a subpopulation of slow proliferation $LGR5^{+}$ intestinal stem cells (Barriga et al., 2017). Although mex3a function is actually unknown, it appears to be associated, somehow, with cellular stem capacity maintenance, probably related with the Wnt/β-Catenin pathway.

1.4. Objetive

The main objective was the characterization of the protein Mex3a role in mice stomach using three study models, a mex3a knockout mice (mex3a$^{-/-}$), gastric cancer cell lines and organoids culture.

The animal model allows a direct visualization and characterization of the effects caused by the lack of the mex3a protein in the gastric epithelium.

Gastric organoid culture was first established and optimized, to be possible the characterization of growth dynamics and differentiation of gastric organoids driven from wild-type and knockout mice. This method supplements the animal model by enabling, in an agile approach, several tests that could influence the gastric units.

The gastric cancer cell lines used, allowed simpler expression studies of mex3a protein, using several growth conditions.

2. Methods and Materials

2.1. Mice

C57BL/6 mice of both sexes, with ages ranging from 14-70 days, were used. Lgr5-EGFP-IRES-CreERT2 (Lgr5) (Barker et al, 2007), Mex3a knockout (Mex3a-/-) (Jackson Labs #010531).

2.2. Isolation of gastric glands

C57BL/6 mice with 3-10 weeks were sacrificed according to ethical procedures of i3S Animal Facility, using isoflurane followed by cervical dislocation. Mice abdomen was washed with 70% EtOH, an incision into the abdominal cavity just to the external genitalia was made and extended to the rib cage by cutting the abdominal musculature on both sides. The stomach was gently pulled out of the abdominal cavity, separated from the intestinal tract by cutting at the level of the duodenum and at the level of the esophagus. The stomach was opened through the greater curvature and the contents were removed by vigorous washing with ice cold PBS. The corpus and antrum regions were separated under a magnifying lamp, cut into 5mm fragments and placed in 15mL falcon tube with 10 mL PBS on ice. The fragments were rinsed with ice-cold PBS 3x with vigorous agitation. Then, the fragments were incubated for 3h in 10mL EDTA 10mM with agitation at 4°C to detach the epithelial layer from the mesenchymal one. From this step onward the procedure was carried out under a laminar flow chamber. The supernatant was discarded and 10mL fresh PBS added. Tissue chunks were pipetted approximately 40 times up and down with a FBS-coated Pasteur pipette to disrupt the tissue and separate the antral/corpus glands from the basal layer. The gland suspension was transferred to a new 50mL falcon tube using a 70µm strainer to remove large chunks of tissue. The remaining glands were resuspended in 5mL fresh PBS and the procedure was repeated twice more. The filtered solution (20mL) containing the glands was centrifuged at 150g, 4°C for 10 min. The majority of the supernatant was removed, leaving approximately 1mL to resuspend the pellet, then the solution was transferred to an eppendorf and centrifuged again at 150g, 4°C for 10 min. The supernatant was fully discarded whereas the pellet containing the gastric glands was further used to establish the organoids.

2.3. Organoid culture

The method used to culture gastric organoids was adapted from (Barker et al., 2010a; Demitrack et al., 2015; Vallone et al., 2017) with modifications. Antral and corpus glands were isolated as described above and the resulting pellets were sharply resuspended in Cultrex® BME 2, Pathclear™ (Amsbio), with 0.02% (v/v) primocin^{TM} (InvivoGen) in a proportion of 40µL by well of a 24 well plate (Sarstedt). The gland suspension was plated in a 24 well plate and incubated at 37°C with 5% (v/v) CO_2 and 99%

humidified atmosphere for 15-30min. After polymerization has been achieved, 500µL of gastric corpus and antrum organoid medium was added to each well and refreshed every 2 days. Antrum organoid medium was composed of Advanced DMEM/F12 (Thermo Fisher) supplemented with 50% Wnt3a conditioned medium (ATCC®, CRL-2647™), 10% Rspondin-1 conditioned medium (kind gift from Calvin Kuo), 10% Noggin conditioned medium (kind gift from Gijs van den Brink), 10% Fetal Bovine Serum (Biowest), 10mM Hepes (Thermo Fisher), 1X glutamax (Thermo Fisher), 10µM Y-27632 (Sigma), 1mM N-acetilcystein (Sigma), 0.02% (v/v) primocin^{TM} (InvivoGen). Corpus organoid medium was similar to the previous one with the addition of 10nM gastrin (Sigma). Organoid plate passage was performed every 7-8 days (depending on the rate of growth). Organoid passage was carried out by medium aspiration, followed by the addition of 1mL cold PBS to each well. Matrix disruption and detachment was achieved by pipetting up and down the PBS, approximately 20x times. The resulting homogeneous suspension was transferred to a 15mL conical tube (Sarstedt). A centrifugation was made for 10min at 4°C and 800g. Supernatant was discarded and 300µL cold PBS added. Organoid mechanical disruption was achieved by pipetting up and down with a micropipette (P200) until visual disappearance of big pieces, approximately 40x times. The suspension was washed with 5mL PBS and centrifuged for 10min at 4°C and 800g. The supernatant was removed leaving 1mL to resuspend the pellet. The suspension was transferred to an eppendorf tube and centrifuged for 10min at 4°C and 800g. At this point the supernatant was fully removed and the pellet was resuspended in 0.02% (v/v) primocin cultrex solution which allowed a dilution of the initial material from 2 wells to 3. The disrupted organoid suspension was plated in a 24 well plate and incubated at 37°C with 5% (v/v) CO_2 and 99% humidified atmosphere for 15-30min and cultured as previously described.

2.4. Gastric cell lines

Gastric cancer cell lines AGS (ATCC®, CRL-1739™), MKN45 (Riken, RCB1001), MKN28 (Riken, RCB1002), SNU-638 (Korean Cell line bank,) and NCI-N87 (ATCC, CRL-5822™) were grown in Roswell Park Memorial Institute (RPMI) 1640, GlutaMAX™ Supplement, HEPES (Thermo Fisher Scientific) medium supplemented with 10% (v/v) heat inactivated fetal bovine serum (Biowest) and 100 units/mL of penicillin, 100µg/mL of streptomycin, or 1% (v/v) Penicillin/Streptomycin (Thermo Fisher Scientific). Cells were maintained in an incubator at 37°C with 5% (v/v) CO_2 and 99% humidified atmosphere in 75cm^2 culture flasks (Sarstedt). For cell passage, the medium was aspirated and the cells were washed with 5mL of pre heated PBS. Three mL trypsin (Thermo Fisher Scientific) were added and flasks were incubated at 37°C for 5-10min. To confirm that cells were detached from the flask surface, the flasks were observed in an inverted microscope and finally 1mL FBS was added to obtain trypsin inhibition. This step was followed by a centrifugation at 1200 RPM for 5 min, the supernatant was

discarded and the cells were resuspended in culture medium and cultured in new T75 flasks. This process was repeated every 3-5 days and depending on the cellular density, different dilutions were applied.

2.5. Preparation of conditioned media

For Wnt3a conditioned medium production, mouse transgenic fibroblasts cells (ATCC®, CRL-2647™), were cultured in simple Dulbecco's Modified Eagle's Medium glutamine supplemented (Thermo Fisher Scientific) on a T75 flask. The cells were grown for 2 days, then passed to two T75 flasks one with simple Dulbecco's Modified Eagle's Medium (DMEM) to maintain the cellular line in culture, and another with Advanced DMEM F12 (Thermo Fisher Scientific). The one with advanced DMEM F12, extracellular medium was collected and substituted at the end of 4 days and cells were grown for more 3 days. In the final of the seventh day, the medium was collected, mixed with the fourth day medium and filtered using a PES Syringe filter with 220nm (Frilabo) pore size, becoming ready to use. The remaining cells were discarded.

2.6. Histology and histochemistry

After collection, mouse stomachs were opened and fixed at least 24h with 10% (v/v) neutral buffered formalin (PanReac) at RT, and processed for 12h in a Microm STP-120 spin tissue processor (Thermo Fisher, USA) to achieve paraffination.

For histochemistry procedures, 3µm tissue sections were obtained followed by deparaffination with 2x xylene (Fisher Scientific UK) for 10 min, and hydration through a series of ethanol solutions (100%, 100%, and 70%) for 3 min each and finally a bath in tap water for 5 min. Modified Mayer's hematoxylin (Thermo Scientific) staining was achieved by 1 min incubation followed by a 5 min current water washing. Eosin counterstaining was carried out by 2/3 min incubation with an alcoholic eosin solution (Thermo Scientific) preceding one wash in 95% ethanol for 3 min. For dehydration step a series of three increasing % (v/v) ethanol solutions were used (95%, 100% and 100%), 3 min each step, ending with 2x xylene solution incubation, for 5 min long. For cover slips mounting medium (Thermo Scientific) was used and air dried for 30min at least.

For periodic-acid Schiff staining, incubation with periodic acid (Thermo Scientific) was performed for 10 min, followed by staining with Schiff's reagent (Thermo Scientific) for 15 min. After these steps the slides were counterstained with hematoxylin, dehydrated and mounted as previously described.

2.7. Immunohistochemistry and immunofluorescence

IHC was performed on deparaffinated and rehydrated 3µm tissue sections, using two different detection systems, Avidin-Biotin complex (ABC) and peroxidase polymer systems.

Antigen retrieval was carried out by heating slides in an IHC-Tek Epitope Retrieval Steamer Set (±100°C) for 40 min either in a modified citrate buffer pH 6(Thermo Scientific) or Tris/EDTA buffer pH 8, (Thermo Scientific). After that, the slides were cooled down for 20min at RT, and washed 1x in Tris-buffered saline (GRISP) with 0.05% (v/v) TWEEN® 20 (Sigma) for 5min. Next endogeneous peroxidase inhibition was performed by incubation in a 3% (v/v) hydrogen peroxide solution (EMsure), for 10min at room temperature (RT), and slides were washed twice in 0.05% (v/v) Tris-buffered saline Tween (TBS-T). For blocking step, unnecessary for peroxidase polymer system, incubation for 30min at RT was made with normal serum of the same animal species as the secondary antibodies were produced (1:5, DAKO). Approximately 100µL of primary antibody solutions per slide were incubated overnight at 4°C. Every dilution was made in antibody diluent OP Quanto (Thermo Scientific).

The ABC system was used with the following primary antibodies: mouse monoclonal anti-ATPase (1:500, Santa Cruz Biotechnology, sc-374094), mouse monoclonal anti-ChgrA (1:50, Santa Cruz Biotechnology, sc-393941), rabbit monoclonal anti-Ki67 (1:1000, Abcam, ab16667). The biotinylated secondary antibodies were rabbit polyclonal anti-mouse (1:100, DAKO, E0354) and swine polyclonal anti-rabbit (1:100, DAKO, E0353). Before secondary antibody incubation, a solution with avidin (1:100, Vector Laboratories) and biotin (1:100, Vector Laboratories) was prepared at least 30min before usage. Secondary antibody incubation was carried out for 30min at RT and next the slides were washed 2x times in 0.05% (v/v) TBS-T. For signal amplification, an incubation with the complex solution of Avidin/Biotin, was carried out for 30min at RT followed by 2x washes in 0.05% (v/v) TBS-T.

The peroxidase polymer system was used with the primary antibody rabbit monoclonal anti-Sox2 (1:50, cell marque, 371R-16). Secondary antibody solution with polymer (DAKO, Denmark) was added to the slides, just enough to cover the tissue, and incubated for 30min at RT. Revelation step of both systems was performed by adding diaminobenzidine (1:50, DAKO) to slides for 5-10 min, following washing for 5 min in tap water.

After these procedures, slides were counterstained with hematoxylin and mounted with mounting medium.

Immunofluorescence was performed with the mouse monoclonal antibody anti-β-catenin (1:100, Santa Cruz Biotechnology, sc-7963). The procedure was similar to immunohistochemistry until primary antibody incubation after which the technique was carried out in the dark. The secondary antibody used was a goat polyclonal anti-mouse conjugated with Alexa fluor ® 594 fluorescent dye (1:100, Thermo Fisher Scientific, A11032). 100µL were added to slides, and incubation lasted 45min, with 2x washes

in 0.05% TBS-T. Cover slips were mounted using VECTASHIELD Mounting Medium containing DAPI (Vector Laboratories) for nuclear staining. The slides were isolated with varnish. Tissues immunostainings were repeated on at least four different animal cases. Only representative immunostainings were included in the manuscript.

2.8. In situ hybridization

All in situ hybridizations were carried out using RNAscope® 2.5 kit (Advanced Cell Diagnostics) and the protocol was according to the manufacturer instructions. Hybridizations were performed in 5µm tissue sections fixed with 4% (v/v) paraformaldehyde in Superfrost™ Plus (Thermo Fisher) slides.

Probes were anti-Mex3a and anti-Olfm4 mRNA (Advanced Cell Diagnostics). All assay reagents belong to the RNAscope® 2.5 kit.

2.9. Protein Extraction

Protein extraction was performed in cultured cells, using a lysis solution (90% (v/v) NP-40 lysis buffer, 1% (v/v) 100mM Phenylmethanesulfonyl fluoride (Sigma, 174.2g/mol), 4% (v/v) 25X cOmplete^{TM} (ROCHE), 4% (v/v) 500mM NaF (Sigma) and 1% 100mM Na_2VO_4 (Sigma)). On ice, cells pellet were resuspended in a proportion of 1mL of lysis solution per 10^8 cells and incubated for 20min, shaking the tubes every 5min. Then digested cells were centrifuged at 17000g for 15min at 4°C, followed by supernatant recoil. Protein quantification was performed using the Pierce^{TM} BCA Protein Assay Kit following the manufacturer's instructions.

2.10. Western Blotting

To protein sample preparation, 30µg of extracted protein was transferred to an eppendorf tube, added dH_20 to fulfill 12µl of volume. After that, 4µl of loading solution (90% (v/v) 4x Laemmli solution, 5% (v/v) bromophenol blue loading buffer (Sigma, 669.96g/mol) and 5% (v/v) 2-marcaptoethanol (Sigma)) was added and incubated for 5min at 95°C. Next 0.75mm thick acrylamide gel was loaded with 16µl of protein sample per well, along with Precision Plus Protein^{TM} Dual Color standards (Bio-Rad) molecular weight marker and ran at 130V for 90min in Tris-Glycine-SDS Buffer (BIO-RAD). To transfer, nitrocellulose membranes (GE Healthcare Life Sciences) were used together with Tris-Glycine Buffer (BIO-RAD) with 20% (v/v) methanol (Thermo Fisher Scientific) as transfer buffer, and ran at 60V for 120min cooled with ice. Ponceau staining (Sigma) was used to confirm protein transference to the membrane. Membrane blocking was carried out with 5% (m/v) Bovine Serum Albumine (Sigma) in 0.1% TBS-Tween solution, for 60min at RT with constant shaking. The primary antibodies were

incubated overnight at 4°C with constant agitation. The primary antibodies used were the following: rabbit polyclonal anti-MEX3A (1:2500, Sigma, PRS4869), rabbit monoclonal Anti-SOX2 (1:500, Cell Marque, 371R-16), rabbit, monoclonal anti-SOX9 (1:1000, Cell Signaling, D8G8H), mouse monoclonal Anti-β-Catenin (1:1000, BD Transduction Laboratories™ , 610153), rabbit monoclonal anti-ACTB (1:8000, Santa Cruz Biotechnology, Sc-1616-R), goat polyclonal anti-ACTB (1:8000, Santa Cruz Biotechnology, Sc-1616), mouse monoclonal anti-CDX2 (1:500, Biogene, MU392A-UC), rabbit monoclonal anti-GLUT1 (1:1000, abcam, ab15309).

After incubation with the primary antibodies, the membranes were washed three times in 0.1% (v/v) TBS-T for 10 min each, at RT on a rocking platform. The following secondary antibodies were employed on the membranes: Goat anti-rabbit (1:2000, Santa Cruz Biotechnology, sc-2004), chicken anti-mouse (1:2000, Santa Cruz Biotechnology, sc-2954) and donkey anti-goat (1:2000, Santa Cruz Biotechnology, sc-2020). Secondary antibody incubation was performed for 1h at RT with constant agitation. The membranes were then washed three times in 0.1% (v/v) TBS-T for 10 min, at RT in a rocking platform and finally revealed with Amersham™ Enhanced ChemiLuminescence™ (GE Healthcare Life Sciences) solution for 5min at RT, followed by signal revelation using revelation and fixation solutions (Carestream Dental) on a Hypercassette™ (Amersham Biosciences) in the dark.

2.11. Transient and stable transfections with Mex3a expression vector

Transfection was carried out in cell lines, by diluting 9µL of lipofectamine (Thermo Fisher) and 6µg of mex3a expression pCMV-MEX3A vector (Buchet-Poyau et al., 2007) in 750µL opti-MEM™ (Gibco). Then the tubes were incubated for 45min at RT and mixed together in equal proportions. For each well of a six-well plate, 500µL of opti-MEM (Thermo Fischer) was added and then 500µL of mixed solution. The medium was changed after 12h to 1mL of RPMI 1640.

For stable transfection, 12h after the first medium exchange the medium was exchanged again to RPMI 1640 with 600µg/mL of geneticin (Thermo Fisher) (vector's resistance mark). Cells were kept in culture for two weeks under these conditions, and then the geneticin concentration was altered to half, just to maintain a selective pressure.

3. Results

3.1. *Mex3a* mRNA expression pattern in the stomach

We started this work by studying the *Mex3a* mRNA expression pattern in major organs of C57Bl/6 mice by RNA *in situ* hybridization.

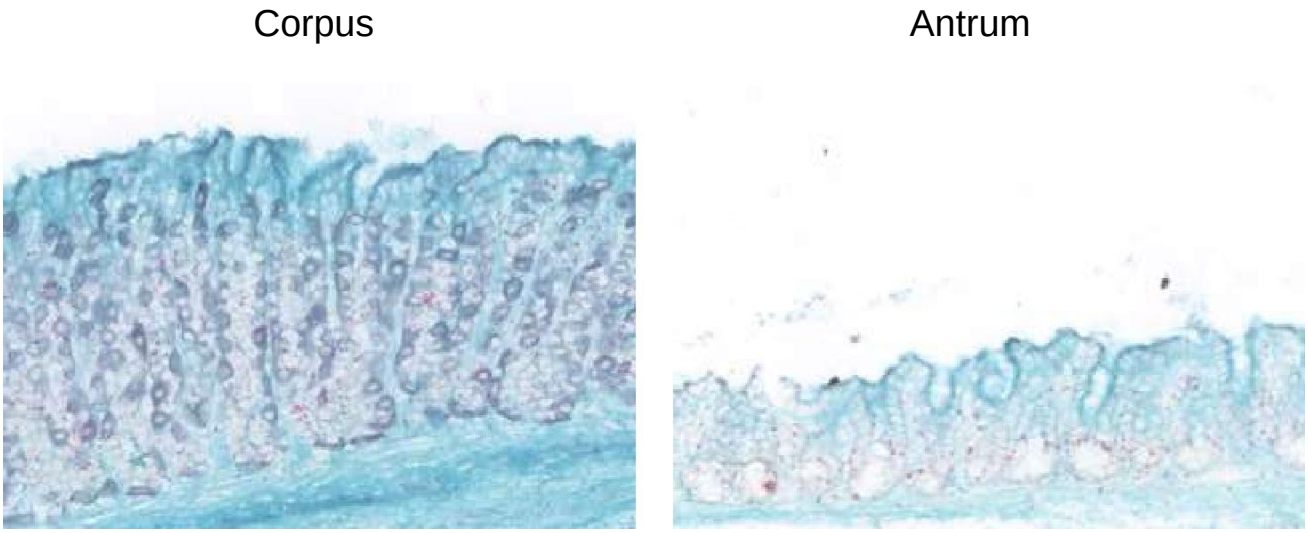


Figure 3. 1 *Mex3a* mRNA expression in gastric corpus and antrum. Each red dot depicts a hybridization event against a single *Mex3a* mRNA molecule (original magnification, x200).

Mex3a mRNA expression was detected in several tissues including intestine, brain, mammary gland, skin, thymus, reproductive organs (data not shown) and also stomach (Fig. 3.1). In the corpus, *Mex3a* mRNA appears to be dispersed along the glands, being absent from the foveolar epithelium, while in the antrum, *Mex3a* mRNA appears to be more expressed at the base of the glands. These observations suggest that there is a biological context for a putative MEX3A function in gastric stem cells, particularly in the antrum, given the overlap in location with other putative stem cell markers, namely LGR5.

3.2. *Mex3a* knockout mice general characterization

In an effort to characterize the physiological role of *Mex3a* in different biological contexts, including the stomach, we characterized mice having a targeted intragenic deletion of the *Mex3a* locus coding sequence, developed under the framework of the INFRAFRONTIER-I3 European research infrastructure. The deletion cassette consisted of a *LacZ* reporter cDNA followed by a floxed promoter-driven neomycin (*Neo*) resistance gene that was removed after crossing with the *Meox2*$^{+/Cre}$ deleter strain (Fig. 3.2A).

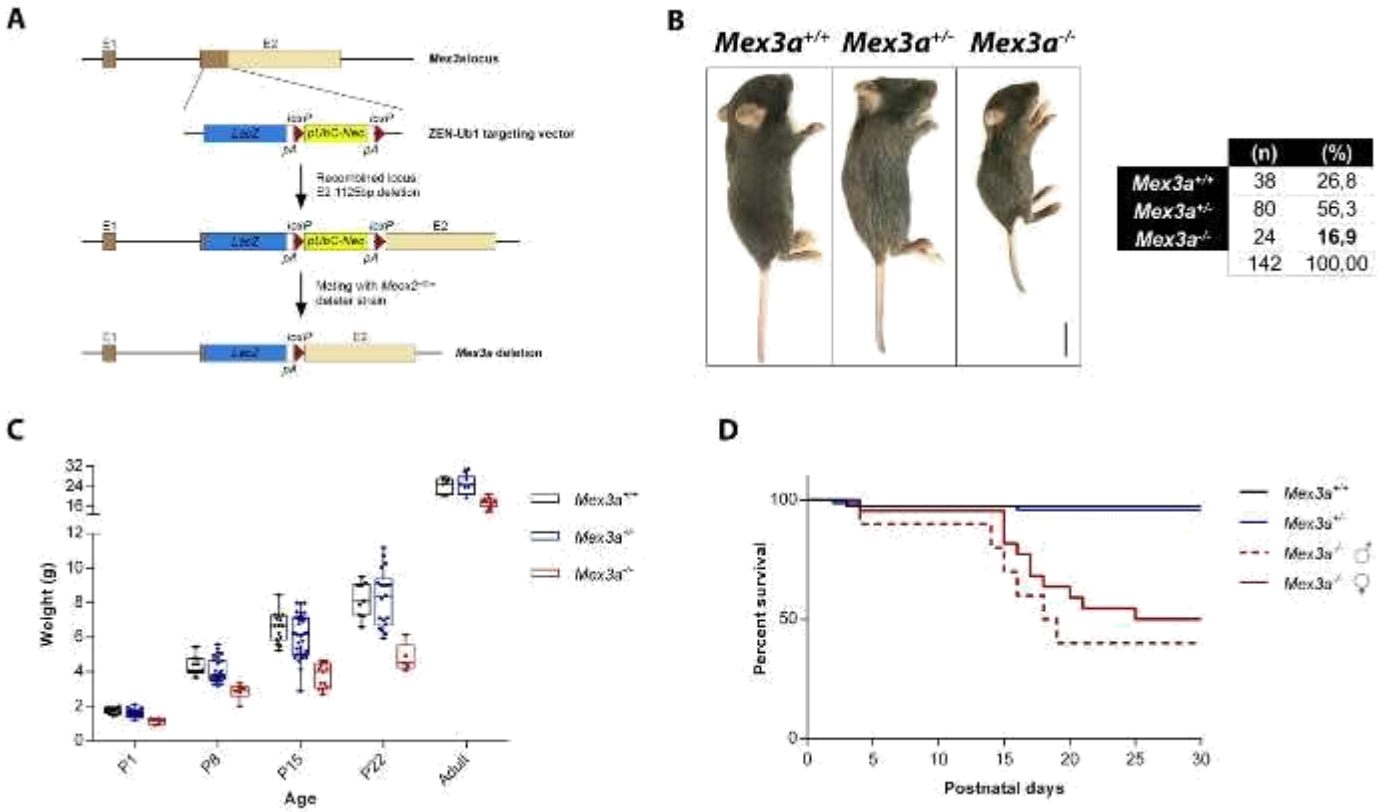


Figure 3. 2 *Mex3a* wild-type, heterozygous and knockout mice analysis and characterization. (A) Schematic representation of the *Mex3a* locus deletion in mouse embryos. (B) In the left panel, a mouse is represented with each genotype. In the right panel the percentages of mice born with each of the three genotypes is indicated. (C) Graphical representation of the total weight of each mouse according to genotype and age (P – postnatal day). (D) Graphical representation of the survival percentage of the mice born with each of the three genotypes

Mex3a$^{-/-}$ homozygous (KO) animals were not born at the expected Mendelian frequency (17% observed versus 25% expected), indicating partial embryonic lethality (Fig. 3.2B). The ones that survived were smaller than their wild-type (Wt) or *Mex3a*$^{+/-}$ heterozygous (Het.) siblings since birth (Fig. 3.C). Although smaller, their weight gain was proportional to *Mex3a*$^{+/+}$ and *Mex3a*$^{+/-}$ animals until postnatal day (P)13 (data not shown). From this point onwards, around 60% of these animals presented progressive weight loss, lethargy, and dehydration signs between P14 and P25, eventually culminating in death (Fig. 3.2D). These results demonstrate that the *Mex3a* gene, although with an incomplete penetrance, has an essential function for murine homeostasis.

3.3. Stomachs of mex3a knockout mice exhibit an epithelial phenotype

Whereas other major organs are being studied in the context of other projects, in this one we focused on characterizing the phenotype of the gastric mucosa in Mex3a-/- mice. The stomachs of 27 mice

(7 *Mex3a*$^{+/+}$, 5 *Mex3a*$^{+/-}$, and 15 *Mex3a*$^{-/-}$) were analyzed by immunohistochemistry and immunofluorescence. Only the cases with the best possible tissue orientation were chosen to be represented. Five molecular markers were analyzed, β-CATENIN, SOX2, KI67, H^+/K^+ ATPase and CHGRA. Mucin-expressing cells present in the foveolar epithelium were also observed by PAS reaction staining (Fig. 3.3 and supplementary fig. 7.1).

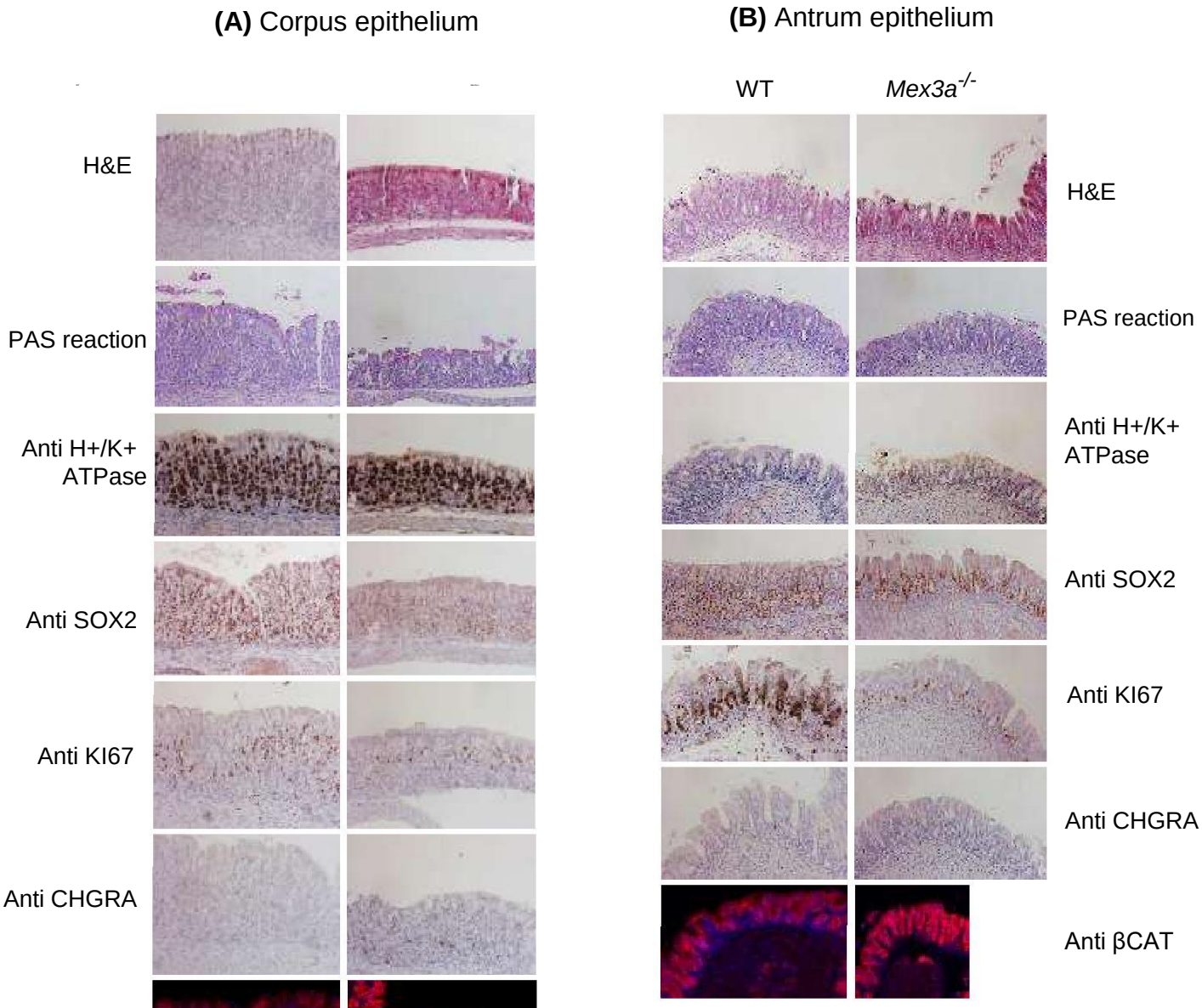


Figure 3. 3 Characterization of stomach tissue from 14-day-old mice. Hematoxilyn & eosin staining (H&E), periodic acid-schiff staining, IHC and IF were used for the labeling of 3µm tissue sections (original magnification, x200). (A) Corpus epithelium representation. On the left side are depicted the staining methods and antibodies employed for IHC and IF. (B) Antrum epithelium representation. On the right side are depicted the staining methods antibodies employed for IHC and IF.

The gastric epithelial phenotype of *Mex3a*$^{-/-}$ mouse is more pronounced in the corpus epithelium. The thickness of the mucosa from knockout mice appears to be diminished, particularly in the corpus (Fig. 3.3A). In the antrum (Fig. 3.3B), esophagus (Supplementary Fig. 7.2A) and forestomach (Supplementary Fig. 7.2B) the tissue is thinner than the corpus, but similar between WT and *Mex3a*$^{-/-}$. Regarding the markers in both mice of each genotype, no differences is observed, with exception for KI67+ cells that appear to be decreased in both the antrum and corpus of 14-day-old and 25-day-old mice knock-out mice (Fig. 3.3A and B and Supplementary fig. 7.1), suggesting that Mex3a-/- gastric cells have lower proliferative potential. These observations suggest that MEX3A could have a putative role in gastric stem cell regulation.

3.4. Gastric organoids: culture establishment and optimization

The culture of gastric organoids proved to be much more challenging than initially expected. Various extraction procedures and culture methods were tested in order to achieve organoid formation. The greatest drawback found was in the organoid passage which was probably due to poor stem cell maintenance. The first culture attempts also resulted in contamination with bacteria, which was solved with the replacement of the initial antibiotics by Primocin^{TM}, an antimicrobial agent for primary cells.

Organoids were grown from *Mex3a*$^{+/+}$, *Mex3a*$^{+/-}$, *Mex3a*$^{-/-}$, *Lgr5*$^{+/EGFP}$, *Mex3a*$^{+/-}$;*Lgr5*$^{+/EGFP}$ and *Mex3a*$^{-/-}$;*Lgr5*$^{+/EGFP}$ mice with ages between 21-60 days.

The first gastric organoids to be characterized were from a *Mex3a*$^{-/-}$ mouse and his sibling *Mex3a*$^{+/+}$; *Lgr5*$^{+/EGFP}$ (Supplementary Fig.7.3). For gland extraction, strong mechanical agitation of pieces of stomach tissue was used, but this method exhibited less efficiency than forcefully pipetting up and down (Fig. 3.4A and B). For plating, Intesticult^{TM} mixed with matrigel and culture medium was employed. Corpus organoids were passed once (Supplementary Fig.7.3A) but antral organoids could not be passed (Supplementary Fig. 7.3B). After this try, Intesticult was deemed not suited for gastric organoid culture and no longer used. Until the culture conditions were optimized, in the first two days of culture the organoids growth rate, was always similar regardless of the mouse genotype, the culture method used (matrigel with growth factors or without growth factors), and the culture medium provided (recombinant Wnt3a or Wnt3a conditioned medium and addition or not of FBS) (Supplementary Fig. 7.4).

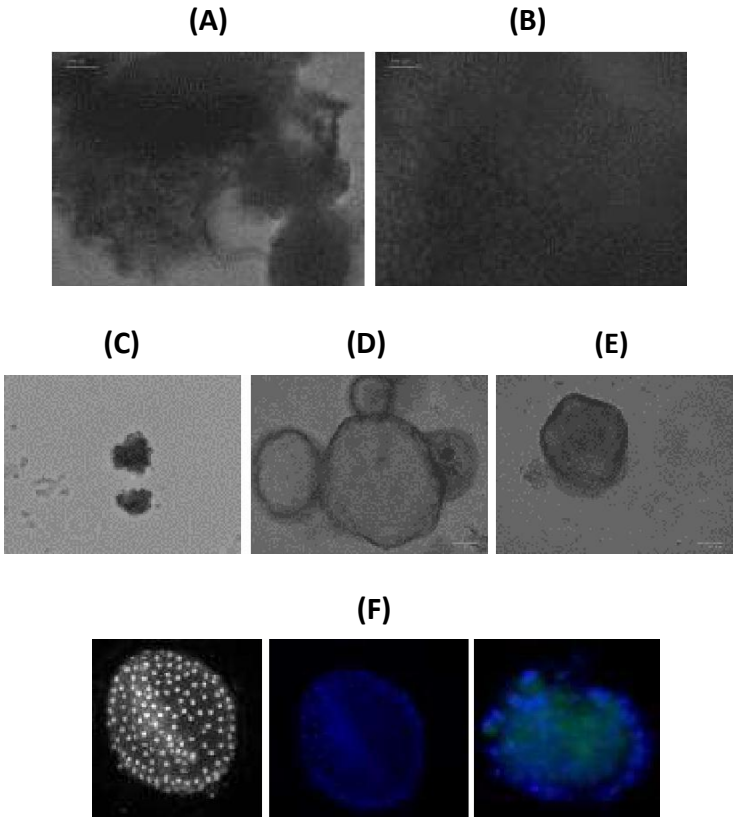


Figure 3. 4 Images set of stomach tissue and gastric organoids. Antral tissue after gland extraction by tissue agitation method **(A)** or tissue pippeting **(B)**. Every hole corresponds to a extracted gland. **(C)** Disaggregated organoids that entered into apoptosis. **(D)** An antral organoid with 6 days in culture from a *Mex3a*$^{+/+}$ mouse. **(E)** Antral organoid with 3 days in culture after the second passage, from a wild-type mouse. **(F)** Immunofluorescence of antral organoids derived from a *Lgr5*$^{+/EGFP}$ mouse with 8 days in culture. Blue color is for DAPI and green for LGR5-EGFP (Original magnification, x100 for the first two images and x200 for the others, scale bar 100µm).

The first three experiments of organoid culture (Supplementary Fig. 7.4A) were plated with the growth factors (recombinant, Wnt3a FGF10 and EGF) mixed in matrigel and culture medium supplemented with N2 and B27. At the end of 4 days the organoids disaggregated and entered apoptosis (Fig. 3.4C). The organoids from experiment #4 (Supplementary Fig. 7.4A) were the first grown with Wnt3a conditioned medium instead recombinant Wnt3a. Some organoids exhibited fluorescence (Fig. 3.4F), suggesting that LGR5^{+} stem cells were present. These organoids entered apoptosis around the 5th day of culture.

Considering that the time in culture could be too long, experiment #5 was carried out (Supplementary Fig. 7.4A). In this instance, growth factors were not mixed in matrigel, just in the medium. EGF, FGF10, N2 and B27 were excluded from the culture medium and replaced by FBS. At the 5th day of culture a passage was made. When passed and re-plated, organoids did not re-grow.

The glands that originated the organoids from *Mex3a*$^{+/+}$ mouse (Supplementary Fig. 7.4A) were the first to be extracted using the method described in 2.2 (Fig. 3.4B). The extraction efficiency was better, and the first culture originated more organoids and larger than the last ones, being more pronounced in the

antrum (Fig. 3.4D). Organoid passage was carried out by the 6th day, using the method described in 2.3 to dissociate them. Some organoids re-grew, but eventually entered apoptosis. The last modification made was the replacement of Wnt3a conditioned medium by another batch where the Wnt3a producing L-cells were grown in the presence of 10% FBS during conditioning. With this new batch, the first successful organoid passage was achieved (with low efficiency) (Fig 3.4E).

3.5. Characterization of gastric organoids from knockout mice

The low birth frequency of knockout mice and the lengthy optimization of gastric organoid culture allowed only the establishment of gastric organoids from one *Mex3a*$^{-/-}$ mouse. The growth rate was the only parameter evaluated (Fig. 3.5A and B).

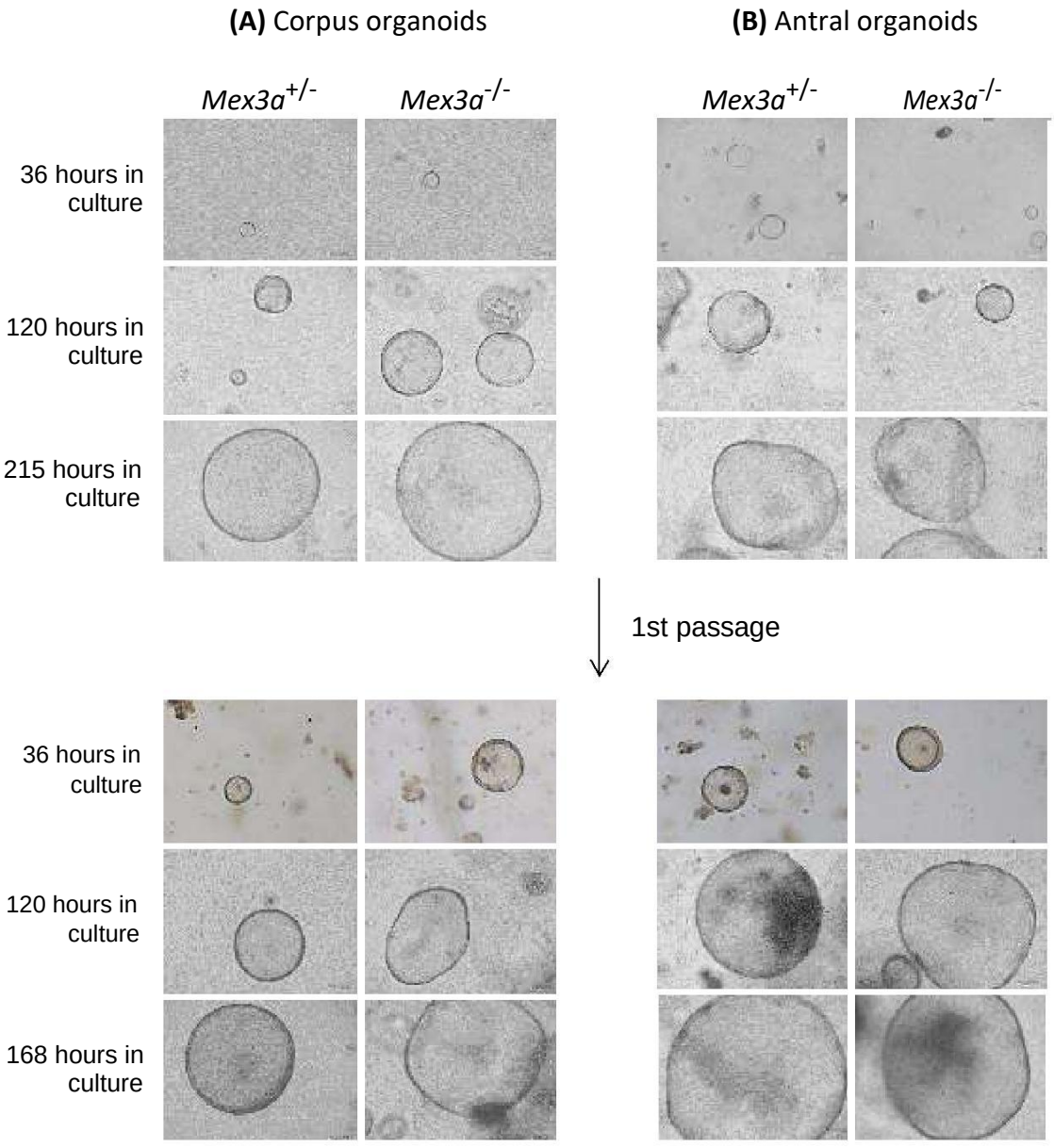
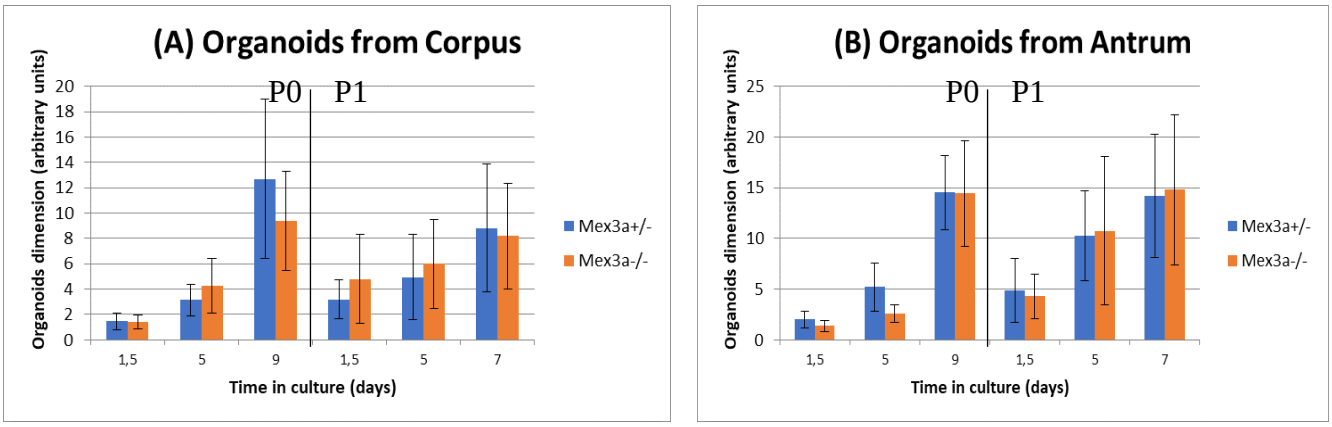
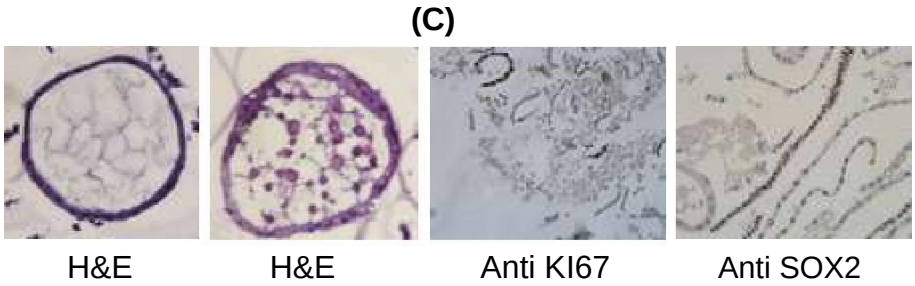


Figure 3. 5 *Mex3a*$^{-/-}$ and *Mex3a*$^{+/-}$ mice derived organoids. On the left side is depicted the organoids age when the photo was taken. (A) Culture of the organoids derived from corpus, *Mex3a*$^{-/-}$organoids exhibited larger dimensions and a faster growth rate during the first culture days than *Mex3a*$^{+/-}$ organoids. (B) Culture of the organoids derived from antrum, *Mex3a*$^{-/-}$organoids exhibited smaller dimensions and a slower growth rate during the first culture days than *Mex3a*$^{+/-}$ organoids.





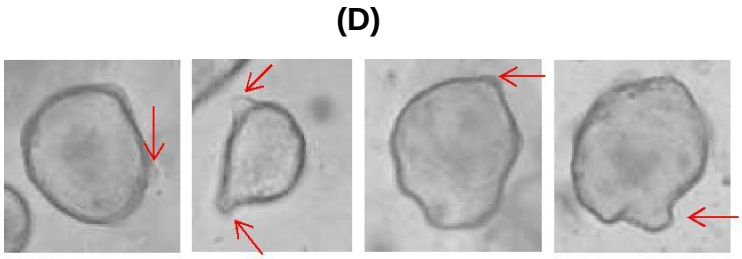


Figure 3. 6 Gastric organoids from *Mex3a*$^{+/-}$, *Mex3a*$^{-/-}$ and double mutant *Mex3a*$^{-/-}$:*Lgr5*$^{+/Egfp}$. (original magnification, x200, scale bar 100µm). **(A)** Graphical depict of *corpus* organoids dimensions evolution during the 9 days after first platement and the 7 days after the second platement (1st passage). **(B)** Graphical depict of *antral* organoids dimensions evolution during the 9 days after first platement and the 7 days after the second platement (1st passage). **(C)** H&E of a *Mex3a*$^{-/-}$ mouse *corpus* organoid (left) and a *Mex3a*$^{+/-}$ mouse *antrum* organoid (right). IHC for the detection of KI67 and SOX2 proteins in *corpus* organoids from a *Mex3a*$^{-/-}$:*Lgr5*$^{+/Egfp}$ mouse. **(D)** Structures evidenced by red arrows correspond to glandular-like features, formed in *Mex3a*$^{-/-}$ *corpus* organoids (three first images) and *Mex3a*$^{+/-}$ antral organoids (right image), after 7 days of the first passage.

Comparing with *Mex3a*$^{+/-}$ mouse, organoids from Mex3a$^{-/-}$ mouse corpus demonstrated a faster growth between 36 and 120 hours becoming identical from this day onward (Fig. 3.6A). On the other hand, the antral organoids demonstrated a slower growth rate between 36 and 120 hours after initial plating, becoming similar from this day onward (Fig. 3.6B). After passage, the re-growth rate was similar between organoids from both mice, but approximately around the 5th day, organoids started to form cystic structures (Fig. 3.6D) suggesting differentiation. Corpus glands extracted from *Mex3a*$^{-/-}$;*Lgr5*$^{+/EGFP}$ mouse originated organoids that expressed SOX2 and KI67 (Fig. 3.6C).

3.6. MEX3A and SOX2 expression in AGS cells

In order to study the relationship of MEX3A with stem cells, an experiment was performed (Supplementary Fig. 7.5) using gastric carcinoma cell lines grown in medium supplemented with the growth factors (in form of conditioned medium) RSPO1, Nog and Wnt3a to promote stemness. SOX2, SOX9, MEX3A, CDX2 and MEX3A protein expression was evaluated (Fig. 3.7A).

The proliferation rate of AGS and MKN45 cells did not change when grown in supplemented medium, whereas MKN28 cells increased and SNU-638 decreased (Supplementary Fig. 7.5). MKN45 and AGS were the only cell lines with increased SOX2 expression in response to supplemented medium.

Assays with cells grown in medium with separated growth factors were performed. In MKN45, R-SPONDIN1 was the main factor to promote the SOX2 expression increase. On other hand in AGS cells, the presence of both Wnt3a and RSPO1 was needed (Fig. 3.7B). MEX3A expression increased in MKN45 cell when SOX2 increased too, but not in AGS cells (Fig. 3.7C), these results were confirmed at least with two more assays (data not shown).

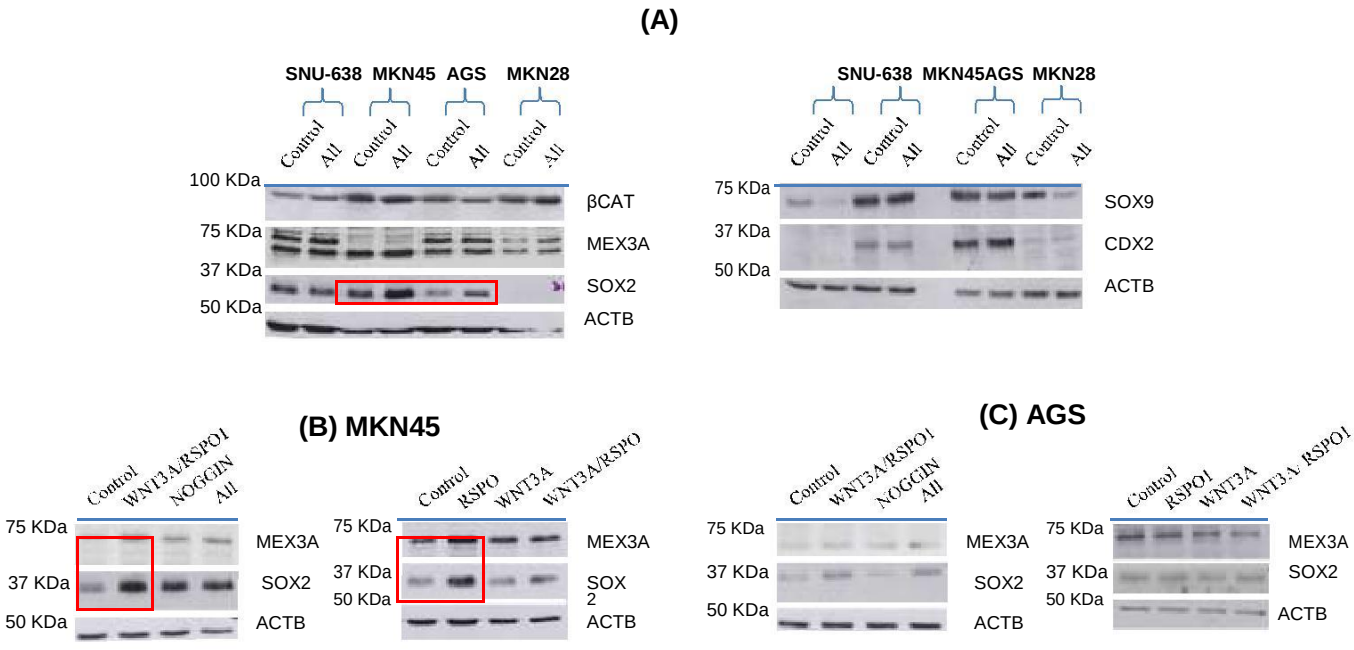


Figure 3. 7 Western blot analysis of SNU-638, MKN45, AGS and MKN28 cell lines. **(A)** Western blot of SNU-638, MKN45, MKN28 and AGS cells grown in normal and supplemented medium. Red box highlights the SOX2 expression increase in AGS and MKN45 cells grown in supplemented medium. **(B)** Western blot of MKN45 cells grown in conditions of separated growth factors, red box highlights the SOX2 and MEX3A expression increase. **(C)** Western blot of AGS cells grown in conditions of separated growth factors.

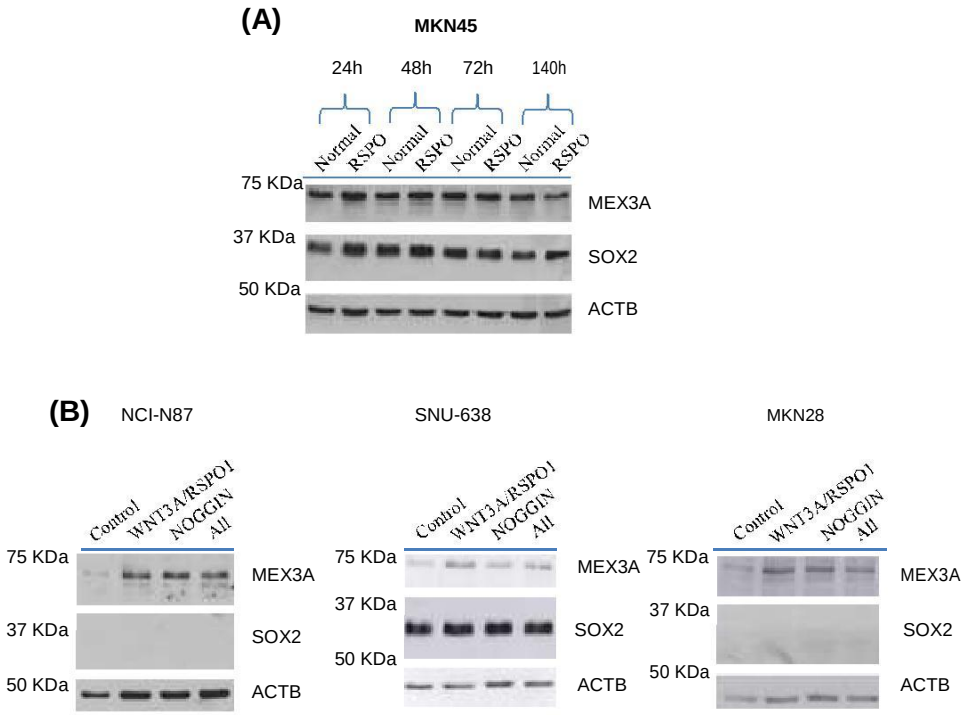


Figure 3. 8 Western blot analysis of several cell lines. **(A)** Western Blot analysis of MKN45 in different time points of cells growth in normal and supplemented medium with RSPO1 for MKN45 and Wnt3a/RSPO1 for AGS. **(B)** Western blot of NCI-N87, SNU-638 and MKN28 grown in supplemented and normal medium.

In MKN45 and AGS cells (data not shown), was determined that 48 hours was enough to increase SOX2 expression (Fig. 3.8A). In SNU-638, NCI-N87 and MKN28 cells SOX2 expression did not increased when cells were grown in supplemented medium, whereas MEX3A expression increased when cells were grown in medium supplemented with all the growth factors (Fig. 3.8B). MEX3A seems like has a role associated with the stemness regulation in these cells.

4. Discussion

MEX3A is an RBP that regulates various targets, including CDX2, responsible for the patterning and differentiation of intestinal *villi* (Pereira et al., 2013). However the MEX3A biological function remains to be fully clarified. To address that, we have generated the first *Mex3a* knock-out mouse that is under characterization.

Mex3a$^{-/-}$ mice were not born at the expected Mendelian frequency (17% observed versus 25% expected), indicating partial embryonic lethality. *Mex3a*$^{-/-}$ mice are smaller than their *Mex3*$^{+/+}$ *Mex3a*$^{+/-}$ siblings since birth. Around 60% of the born *Mex3a*$^{-/-}$ mice presented progressive weight loss, lethargy, and dehydration signs between P14 and P25, eventually culminating in death. This phenotypic variability in penetrance or expressivity is probably due to the knockout allele being present on a mixed genetic background (Doetschman, 2009). Nonetheless, we have already identified a severe intestinal phenotype, which is likely the cause of the post-natal death of 60% of *Mex3a*$^{-/-}$ mice. In this work we sought to investigate if MEX3A has functions in other organs, specifically in the stomach.

Barker et al., demonstrated that a stem cell population, marked by the expression of the membrane receptor LGR5, exists in intestinal crypts and in the base of the gastric antral glands, contributing for the normal epithelium homeostasis (Barker et al., 2007; Barker et al., 2010a). This protein is activated by RSPO1-4 and is a Wnt/β-Catenin pathway agonist, but also accelerates β-catenin and pLRP6 degradation consequently, down regulating Wnt/β-catenin pathway (Carmon et al., 2011). The Wnt/β-catenin pathway plays a central role in adult stem cell regulation and maintenance (Kretzschmar and Clevers, 2017). Recently, Leushacke et al. proved that LGR5 labels a subpopulation of chief cells, resident in the base of gastric corpus glands that acquire stem cell characteristics, following an injury situation (Leushacke et al., 2017).

We started by determining the expression of MEX3A in the mouse stomach. Through ISH, it was observed that MEX3A is expressed at low levels in the base of antral glands and dispersed throughout the corpus glands. These observations suggest that there is a biological context for a putative MEX3A function in gastric stem cells, particularly in the antrum, given the overlap in location with other putative stem cell markers, namely LGR5. Furthermore, since MEX3A is co-expressed with LGR5 in a sub-population of slowly-dividing cells in the intestinal crypts, and these cells can convert into intestinal stem cells, after injury, in order to regain homeostasis (Barriga et al, 2017.), we believed that there was a link between MEX3A and gastric LGR5^{+} cells. Due to the low specificity of LGR5 antibody in IHC, to confirm this putative co-expression a double hybridization with MEX3A and OLFM4 (a protein that labels LGR5-positive stem cells) would be needed (Flier et al., 2009).

Mice stomachs are small organs, namely the ones from *Mex3a*$^{-/-}$ mouse. This characteristic makes the inclusion of stomachs in paraffin (with a noticeable orientation) a tricky process. Nonetheless it was possible to observe that the gastric mucosa from *Mex3a*$^{-/-}$ mice was thinner than normal and heterozygous littermates, particularly in the corpus epithelium. In the forestomach and esophagus this thickness reduction was not observed, suggesting that it is a specific phenotypical manifestation in the corpus, due to the lack of MEX3A. In *Mex3a*$^{-/-}$ mouse, Ki67^{+} cells were decreased in the corpus, but mainly in the antrum. This protein marks cells that exhibit a great proliferative potential (Bullwinkel et al., 2006), suggesting that MEX3A could have a putative role in the regulation of gastric stem cells. A reduction in Ki67^{+} cells suggest less adult stem cells and hence less proliferation.

To assess stem cell function, currently one of the best assays is to generate organoids. Organoids derived from the antrum of *Mex3a*$^{-/-}$ mouse exhibit a slower progression in an initial growing phase. These difficulties suggest a reduction in the number of initial resident stem cells by gland, and are in accordance with the results observed in the tissues, namely decreased number of Ki67^{+} cells. After a few days in culture, the lack of stem cells in organoids could be compensated by the culture medium which is enriched in growth factors, leading *Mex3a*$^{-/-}$ derived organoids to demonstrate similar characteristics as the *Mex3a*$^{+/-}$.

The study in organoids and mice models was complemented by experiments performed in gastric cell lines. To support the suggestion that MEX3A is related with gastric stem cell function, five gastric cancer cell lines were analyzed. Several proteins were studied, being the main focus the MEX3A and SOX2. SOX2 interacts with β-Catenin, promoting the activation of the Wnt/β-Catenin pathway (Chen et al., 2008). This protein also marks gastric stem and progenitor cells in gastric glands, and cancer stem cells (Arnold et al., 2011; Lenglez et al., 2014). Both MKN45 and AGS cell lines increased expression of SOX2 marker when cells were grown in medium supplemented with growth factors which promote Wnt/β-catenin pathway siganling. LGR5 and Wnt ligands are essential for adult intestinal and gastric stem cell maintenance, and consequently for the formation of intestinal and gastric organoids derived from these cells through Wnt/β-catenin activation (Barker et al., 2010a; Sato et al., 2009). In AGS, the increase in SOX2 was the highest when cells were cultured with Wnt3a and R-SPONDIN1. On the other hand in MKN45 cells, R-SPONDIN1 was the main growth factor to contribute to SOX2 expression. The increase in SOX2 expression suggests an increment in stem cell population. NOGGIN appears to not contribute to SOX2 expression increase as was expected, since NOGGIN is a BMP inhibitor and not a Wnt/ β-Catenin activator. In MKN45 and AGS, 48 hours of growth was enough time to observe a SOX2 expression increase. Since SOX2 transcription factor is associated with stemness, the increase of MEX3A, when SOX2 is increased too, suggests a relationship of MEX3A with stemness regulation in MKN45 cells.

The optimization process of gastric organoid establishment was very challenging. In the optimization process (the extraction method, number of glands plated, composition of the matrigel and culture medium and time of growth) one condition was changed at a time. In the first attempts, the commercial intesticult was used as culture medium. If it worked, it would constitute a faster way to establish the culture, because the medium is ready to use and its constitution, being commercial, is more homogenous and controlled than using growth factors supplementation. This medium is more suitable for intestinal organoids, that do not need Wnt3a because they form paneth cells that secrete Wnt3a (Sato et al., 2011).

To overcome this problem, cultures with recombinant Wnt3a present in the culture medium were carried out. Recombinant Wnt3a was not enough to sustain organoids until passage, probably due the high demand for this growth factor. To overcome this limitation, Wnt3a conditioned medium was used. Initially, organoids were not grown with FBS supplement, and not to add a new condition in the organoids culture optimization, L-cells were also grown in medium without FBS. However, we determined that L-cells must be grown with FBS in order to produce an efficient conditioned media (Supplementary Fig. 7.7). Our results clearly indicate that gastric organoids are highly-dependent on the
Wnt/β-catenin pathway signaling, as described before (Barker et al., 2010b; Stange et al., 2013).

5. Conclusion and Future Perspectives

MEX3A is expressed in the stomach, and *Mex3a*$^{-/-}$ mice have a thinner gastric epithelium and less Ki67^{+} cells namely in the antrum. Organoids derived from the antrum of *Mex3a*$^{-/-}$ mouse exhibit a slower progression in an initial growing phase. Taken these demonstrations together, MEX3A has a putative role in LGR5^{+} stem cells regulation probably through Wnt/β-Catenin pathway signaling. This suggestion is corroborated by the assays carried out in the gastric cell lines. MEX3A expression is up-regulated, (with exception of AGS cells) when cells are grown in medium supplemented with growth factors that enhance stem cell maintenance, through Wnt/β-Catenin pathway activation. SOX2 expression (a stem cell marker) is also increased in the same conditions that MEX3A suggesting an increment in stem cells accompanied with MEX3A expression up-regulation.

The link between MEX3A and stemness regulation is still missing. To explore this relationship, it will be necessary to quantify the number of parietal, endocrine, proliferative and SOX2^{+} cells in the stomach of *Mex3a*$^{+/+}$ and *Mex3a*$^{-/-}$ mouse, by Fluorescence-activated cell sorting (FACS). These results will confirm that indeed the thinner gastric mucosa in *Mex3a*$^{-/-}$ mouse is due to less proliferative potential.

More analysis of *Mex3a*$^{-/-}$ stomachs by IHC, ISH and qPCR will be necessary. IHC, ISH and qPCR of corpus and antrum organoids in the first days in culture and after passage would be needed.

Performing IHC and ISH in gastric organoids, derived from *Mex3a*$^{+/+}$ and *Mex3a*$^{-/-}$ mouse, will be necessary to evaluate the differentiation potential of both. Experiments of MEX3A over-expression in *Mex3a*$^{+/+}$ and *Mex3a*$^{-/-}$ organoids, using viral transfection, would assist and complement the characterization of MEX3A role, in gastric epithelium. RNA sequencing of the gastric glands could clarify which pathways are altered, when MEX3A lacks in mouse.

6. References

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7. Supplementary figures

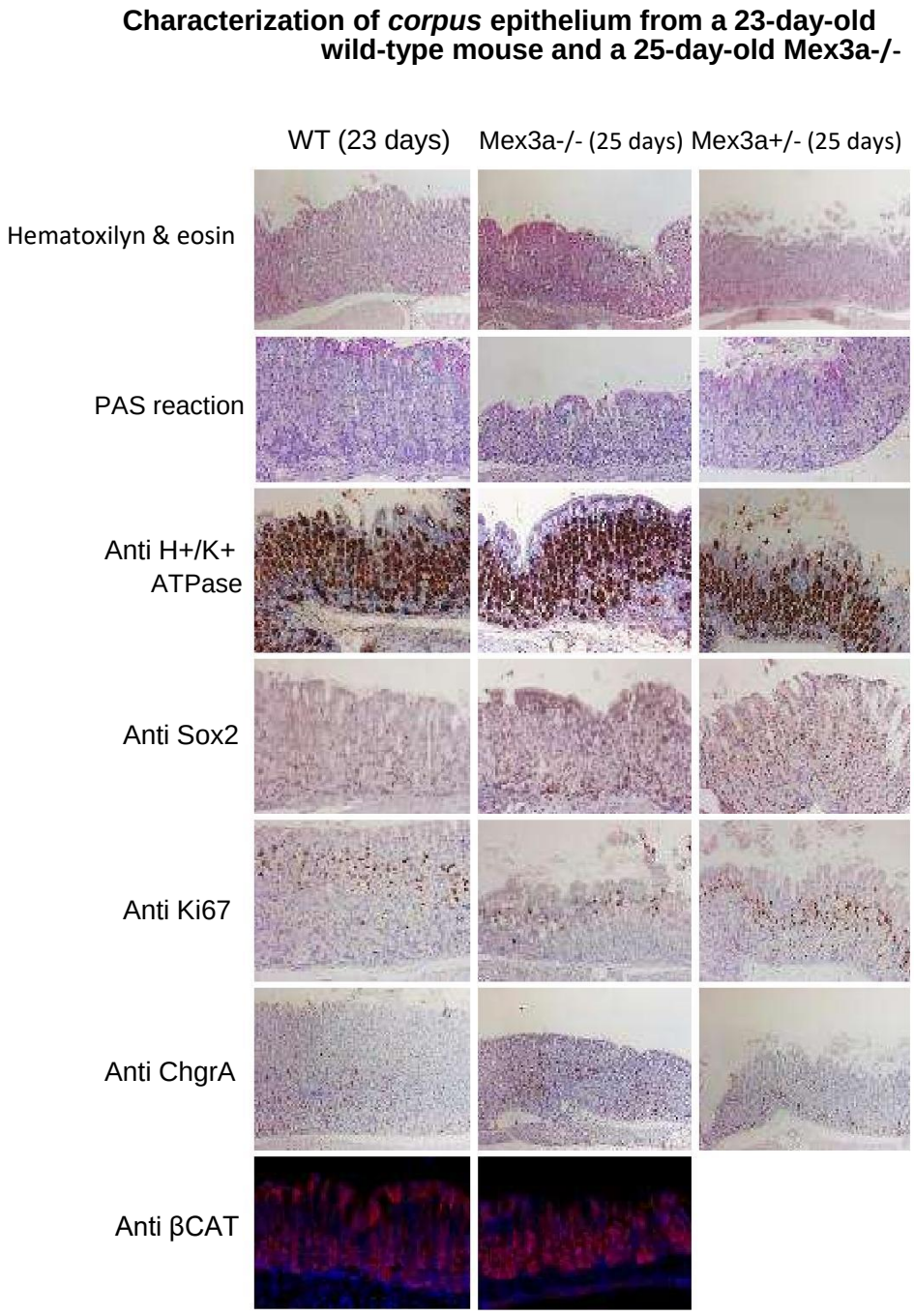


Figure 7. 1 Characterization of corpus tissue from a 23-day-old WT mouse and 25-day-old KO and Het mice, by hematoxilyn & eosin staining, periodic acid-schiff staining, IHC and IF, labeling of 3µm tissue sections (original magnification, x200). On left side is depicted the staining method and antibodies employed for IHC and IF.

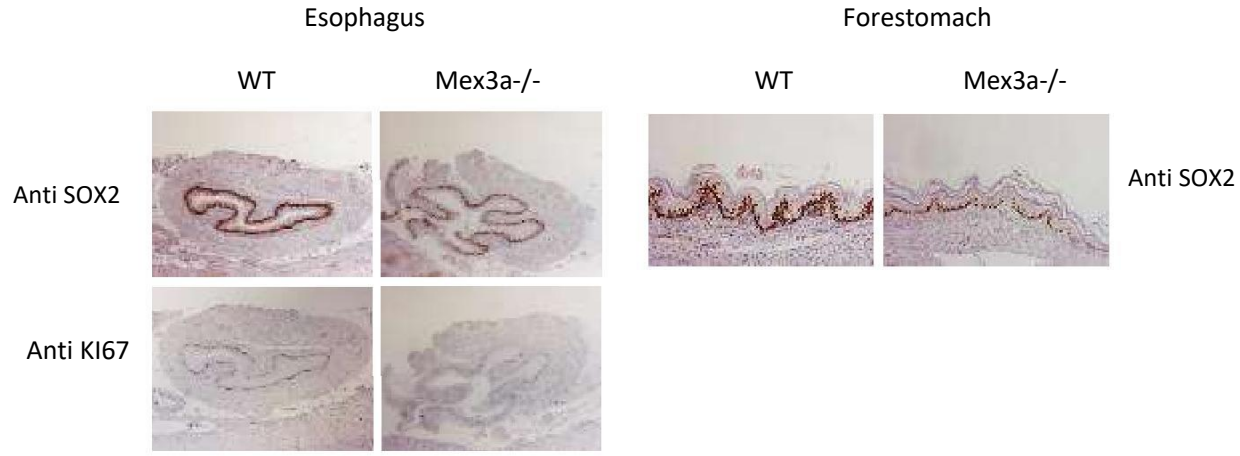


Figure 7. 2 Labeling of 3µm tissue sections from 14-day-old mice by IHC for forestomach and esophagus characterization (original magnification, x200). Esophagus expresses KI67 and SOX2 of the markers analyzed and the forestomach expresses SOX2. On the left and right side are depicted the antibodies employed for IHC.

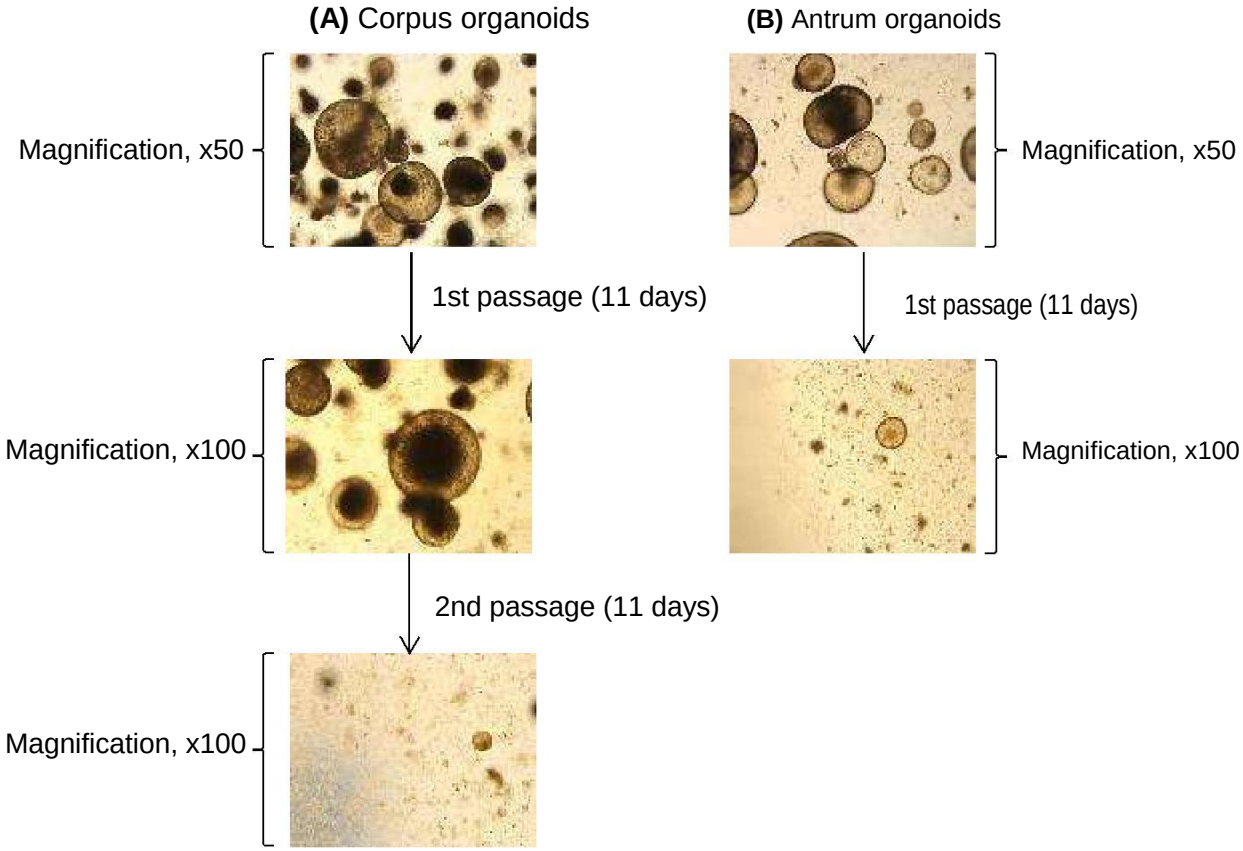
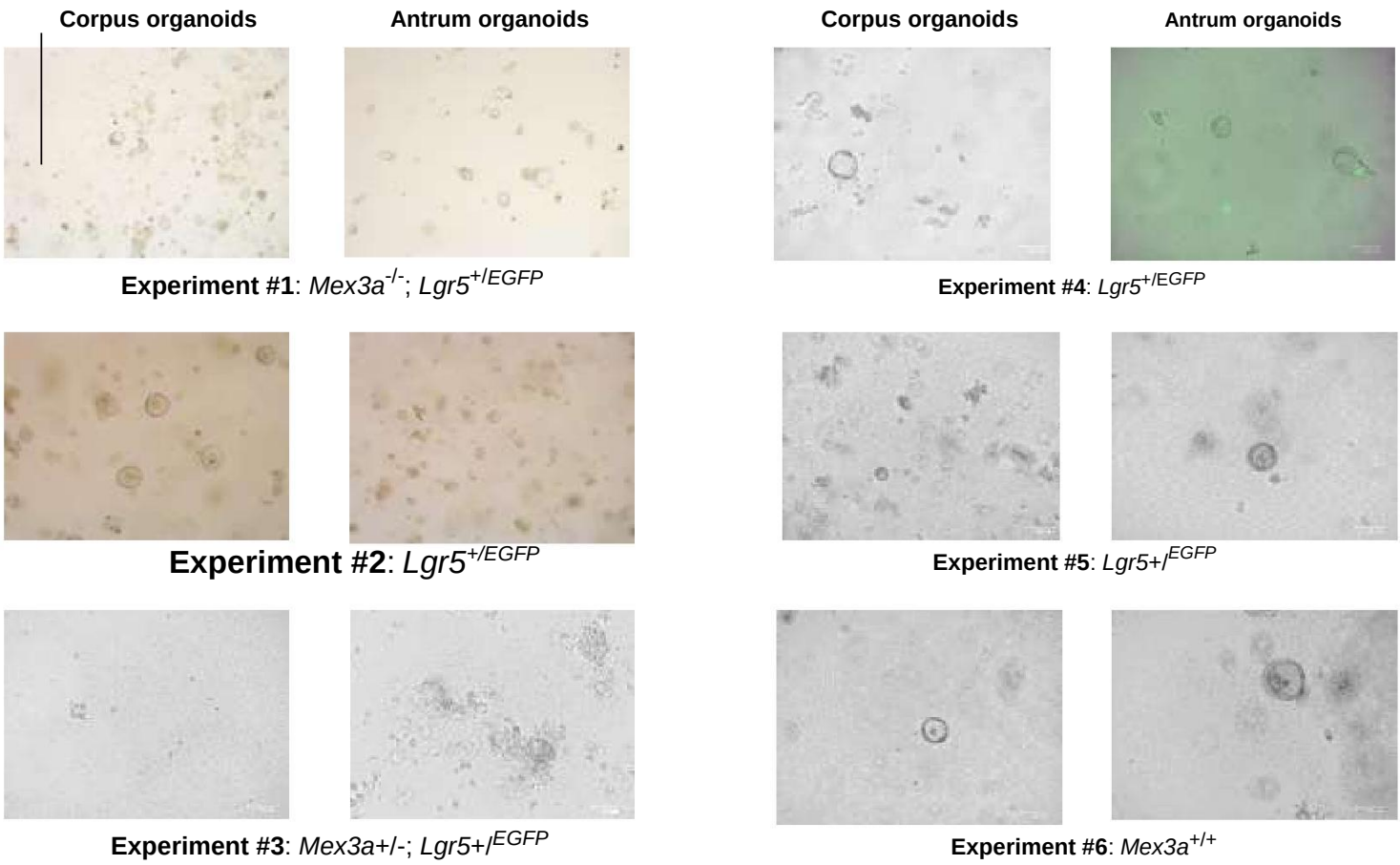


Figure 7. 3 *Mex3a*$^{-/-}$; *Lgr5*$^{+/EGFP}$ mouse derived organoids. All the images were taken on the 11th organoid culture day, starting from day 0 when passed. **(A)** Culture of the organoids derived from corpus, which lasted for 22 days until organoids enter in apoptosis. **(B)** Culture of the organoids derived from antrum, which lasted for 11 days until organoids enter in apoptosis.

(A)



(B)

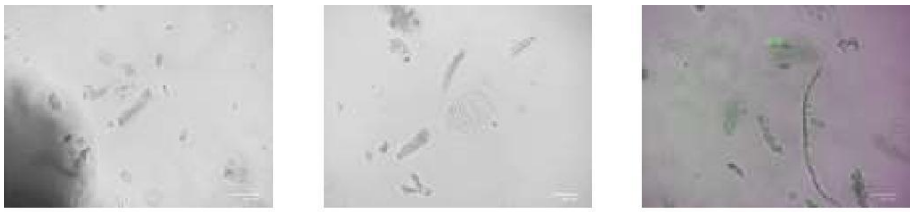


Figure 7. 4 Representative images of gastric organoids and glands isolation. Below the images are represented the experiment number and the genotype of the mouse from where glands were extracted to grow organoids (original magnification, x200, with exception of experiment#1 and the antrum organoids from experiment #2 , x100, scale bar 100µm). **(A)** *O*rganoids grown from different mice with different genotypes, in distinct conditions. In experiment #4 fluorescence was emitted just in antral organoids from *Lgr5*$^{+/EGFP}$ mouse. **(B)** Glands isolated during the extraction process. Fluorescence was emitted just in antral glands from *Lgr5*$^{+/EGFP}$ mouse.

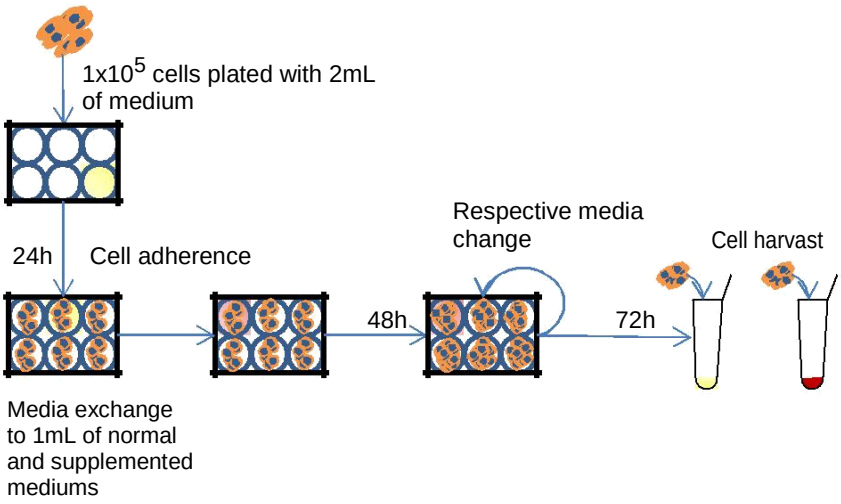


Figure 7. 5 Scheme of cellular lines assay, of growth in supplemented medium. Cell lines SNU-638, AGS, MKN28, MKN45 and NCI-N87 were grown in normal medium and medium supplemented with the growth factors RSPO1, NOGGIN and Wnt3a.

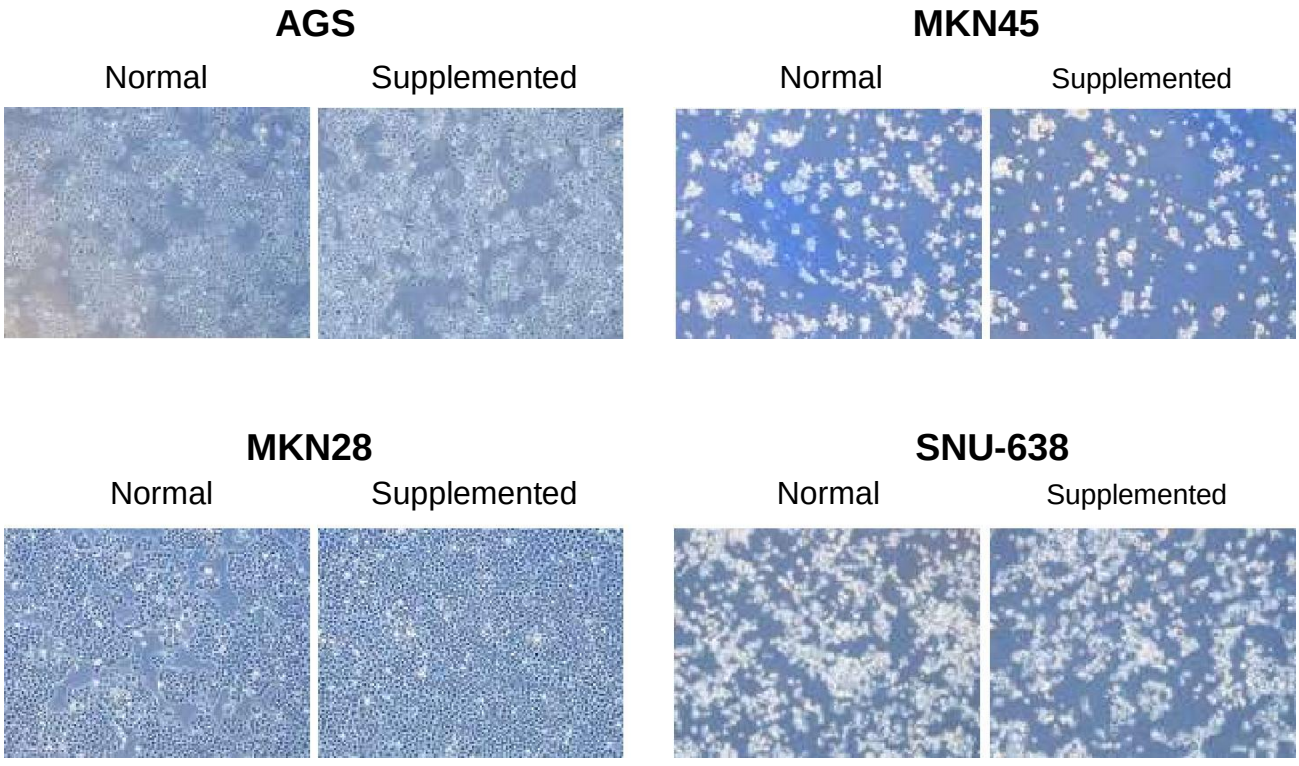


Figure 7. 6 AGS, MKN45 MKN28 and SNU-638 cells grown for three days in supplemented and normal medium (original magnification, x50). Supplemented medium was composed by 10% addition of RSPO1, 10% NOGGIN and 50% Wnt3a conditioned mediums.

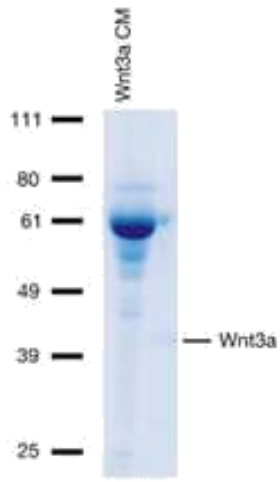


Figure 7. 7 Coomassie staining of an SDS polyacrylamide gel of Wnt3a conditioned medium, collected from mouse L-cells. Size markers are in kilodaltons (Willert et al., 2003).