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Microenviroment modulation on plasticity of

Enteric Nervous System derived cells

Direttore della Scuola: Chiar.ma Prof.ssa MARIA TERESA CONCONI

Coordinatore di indirizzo: Chiar.ma Prof.ssa MARIA TERESA CONCONI

Supervisore: Chiar.ma Prof.ssa MARIA TERESA CONCONI

Co-relatore: Dott.ssa ROSA DI LIDDO

Dottorando: THOMAS BERTALOT

"Imagination is more important than knowledge."
Albert Einstein

Abstract

Growth factors such as EGF, bFGF and GDNF play an essential role in the ENS development and homeostasis. *In vivo* conditions which provide a reduction or absence of these factors promote the development of diseases such as intestinal agangliosis. Thus two models of *in vitro* culture that simulate the physiological condition (SM) and that of agangliosis (BM) was evaluated. ENS-derived cells (ENSc) were isolated from the myenteric plexus of the Sprague Dawley rats [Schaefer et al., 1997]. Particular attention was given to the regulation mechanisms mediated by TLR4 and Wnt signalling. At time of isolation, immunophenotypical characterization by flow cytometry showed the expression of stem cell (SOX2, NANOG, and CD34), neuronal and glial (p75, Nestin, GFAP) markers. Culturing in SM and BM showed a specific modulation of neuronal and glial differentiation and a greater responsiveness mediated by Frizzled 9 (SM) and TLR4 (BM) was observed. Moreover, a neuronal subpopulation co-expressed the receptors TLR4 and Frizzled-9 suggesting that this cell population may be involved in the maintenance of homeostasis and in the regulation of inflammatory processes. Furthermore, only SM cultures formed neurosphere-like structures. Wnt3a stimulation activated the canonical Wnt pathway through Frizzled-9 and qRT-PCR analysis demonstrated anti-inflammatory activity. In addition, a cross-talk between LPS/TLR4 and Wnt pathway was demonstrated by western blotting. Differentiation processes are also influenced by the extracellular matrix (ECM). In this study, the modulatory effect induced by ECM was evaluated assessing an *in vitro* model: ENS-derived cells cultured on a decellularized ECM of adult rat jejunum. Acellular matrixes (AMs) were provided using a modified enzyme detergent decellularization protocol [Meezan et al., 1975]. Histological study, SEM and quantification of residual DNA verified the complete decellularization. Immunofluorescence and western blotting demonstrated that the structural proteins such as collagen I, III , IV and laminin were preserved. After culturing ENSc on AMs for 7 and 14

days, the ECM demonstrated to influence the ENSc spatial organization, exerting a synergic effect with the factors present in the culture medium. In fact, only the AM cultures with SM, showed ganglion-like structures partially interconnected and positive for III tubulin. ENSc cultured on acellular matrix may represent a useful *in vitro* model for toxicological and pharmacological studies as well as a possible tissue scaffold in regenerative medicine.

Riassunto

E' noto che i fattori di crescita quali EGF, bFGF e GDNF giocano un ruolo essenziale nello sviluppo e nell'omeostasi del sistema nervoso enterico (SNE). Condizioni *in vivo* che prevedono un calo o un'assenza del loro apporto, favoriscono lo sviluppo di patologie quali agangliosi intestinale. In questo lavoro di tesi, allestendo due modelli di coltura *in vitro* che simulano la condizione fisiologica (SM) e quella di agangliosi (BM) mediante coltura in presenza (SM) o meno di fattori di crescita (BM) è stata oggetto di studio la risposta differenziativa di cellule isolate da plesso mienterico di ratto Sprague Dawley (ENSc) [Schaefer et al., 1997]. In particolare, veniva prestata attenzione ai meccanismi di regolazione della risposta cellulare mediata dal segnale TLR4 e Wnt. Lo studio di caratterizzazione dell'immunofenotipo mediante citofluorimetria evidenziava nelle popolazioni estratte l'espressione di marcatori di staminalità (SOX2, Nanog e CD34) e di linea neuronale e gliale (p75, Nestina, GFAP). Inoltre, si evidenziava la presenza di una sottopopolazione con caratteristiche neuronali che co-esprimeva i recettori TLR4 e Frizzled-9, suggerendo un ruolo nella regolazione del processo infiammatorio. La coltura in terreno SM e BM dimostrava di modulare in maniera specifica il differenziamento neuronale e gliale delle ENSc e di conferire una maggiore reattività mediata dal Frizzled 9 (coltura SM) e dal TLR4 (coltura BM). Inoltre, l'analisi di microscopia ottica evidenziava la formazione di strutture del tipo neurosfere solo nelle colture trattate con terreno standard. Lo stimolo indotto dal Wnt3a risultava efficace nell'attivare la via di segnale canonica di Wnt attraverso il recettore Frizzled 9 e, all'analisi di espressione genica mediante qRT-PCR, dimostrava un'attività di tipo anti-infiammatorio. Inoltre, mediante uno studio di western blotting, si dimostrava che la via pro-infiammatoria del TLR4 cross-reagiva con il segnale Wnt attivandolo. E' noto che il processo differenziativo è fortemente condizionato dalla matrice extracellulare. In questo studio l'effetto modulatorio indotto dalla matrice sulla risposta differenziativa delle cellule ENSc è stato valutato

utilizzando matrice acellularizzata (AM) di tessuto intestinale di ratto. La preparazione dello scaffold ha previsto 5 cicli ripetuti di decellularizzazione del trattamento modificato detergente enzimatico di Meezan [1975]. La completa decellularizzazione del tessuto veniva verificata mediante studio istologico, analisi di microscopia elettronica a scansione (SEM) e quantificazione del contenuto di DNA residuo. All'analisi di immunofluorescenza e western blotting, le proteine strutturali quali collagene I, III, IV e laminina risultavano preservate al termine della decellularizzazione. Dopo coltura per 7, 14 giorni delle cellule ENSc sulla matrice, AM dimostrava di condizionare l'organizzazione spaziale delle cellule ENSc esercitando un effetto specifico differenziativo in sinergia con i fattori di crescita. Infatti, solo le matrici mantenute in terreno SM mostravano una caratteristica organizzazione delle cellule ENSc in strutture interconnesse di tipo simil-gangliare esprimenti il marcatore neuronale III tubulina. Le colture di ENSc su matrice acellulare possono rappresentare un valido modello *in vitro* per studi tossicologici ed un possibile sostituto tessutale nella medicina rigenerativa.

Table of contents

1 Introduction

1.1 Stemness and lifelong plasticity of enteric nervous system cells

The enteric nervous system (ENS) is the most complex division of the peripheral nervous systems (PNS) in vertebrates. It controls key aspects of gut functionality including the motility, secretion of gastric acid, water and electrolytes and the regulation of blood flow [Furness, 2012]. It is composed of several neurons and glial cells, organized into interconnected ganglia, embedded in the wall of the gastrointestinal tract (Figure 1.1).
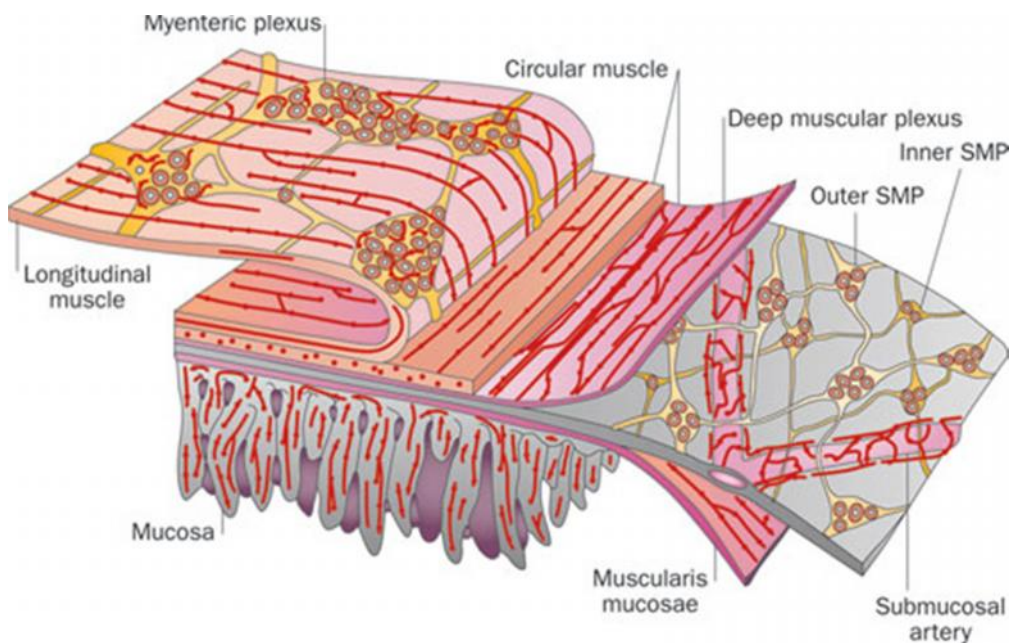
Figure 1.1. The organization of the ENS. The myenteric plexus between the longitudinal and circular layers of the external musculature and the submucosal plexus that is located between the circular muscle and the muscularis mucosae. Nerve fiber bundles connect the ganglia and also form plexuses that innervate the longitudinal muscle, circular muscle, muscularis mucosae, intrinsic arteries and the mucosa. Adapted from [Furness, 2012].

During embryogenesis, ENS development derives from the migration of neural crest cells (NCCs) and involves highly dynamic processes such as proliferation and neuronal and glial differentiation. These mechanisms are under the molecular control of numerous signaling pathways, transcription factors, neurotrophic factors, and the interactions among NCCs and extracellular matrix components [Sasseli et al., 2012]. Undifferentiated

NCCs are initially characterized by high expression of Sox10 (SRY-related HMG-boxgene) [Paratore et al., 2002; Kim et al., 2003]. During the progressing gut colonization an increasing number of NCCs start to upregulate a panel of progenitor marker genes, such as Mash1, Ret, p75, nestin, Phox2b and in some cases of committed neurons (positive for -tubulin III, PGP9.5, HuC/D) or, with slight delay, glial cells (positive for B-FABP, S100, Sox10) [Young et al., 2003; Ruhl, 2005] (Figure 1.2).

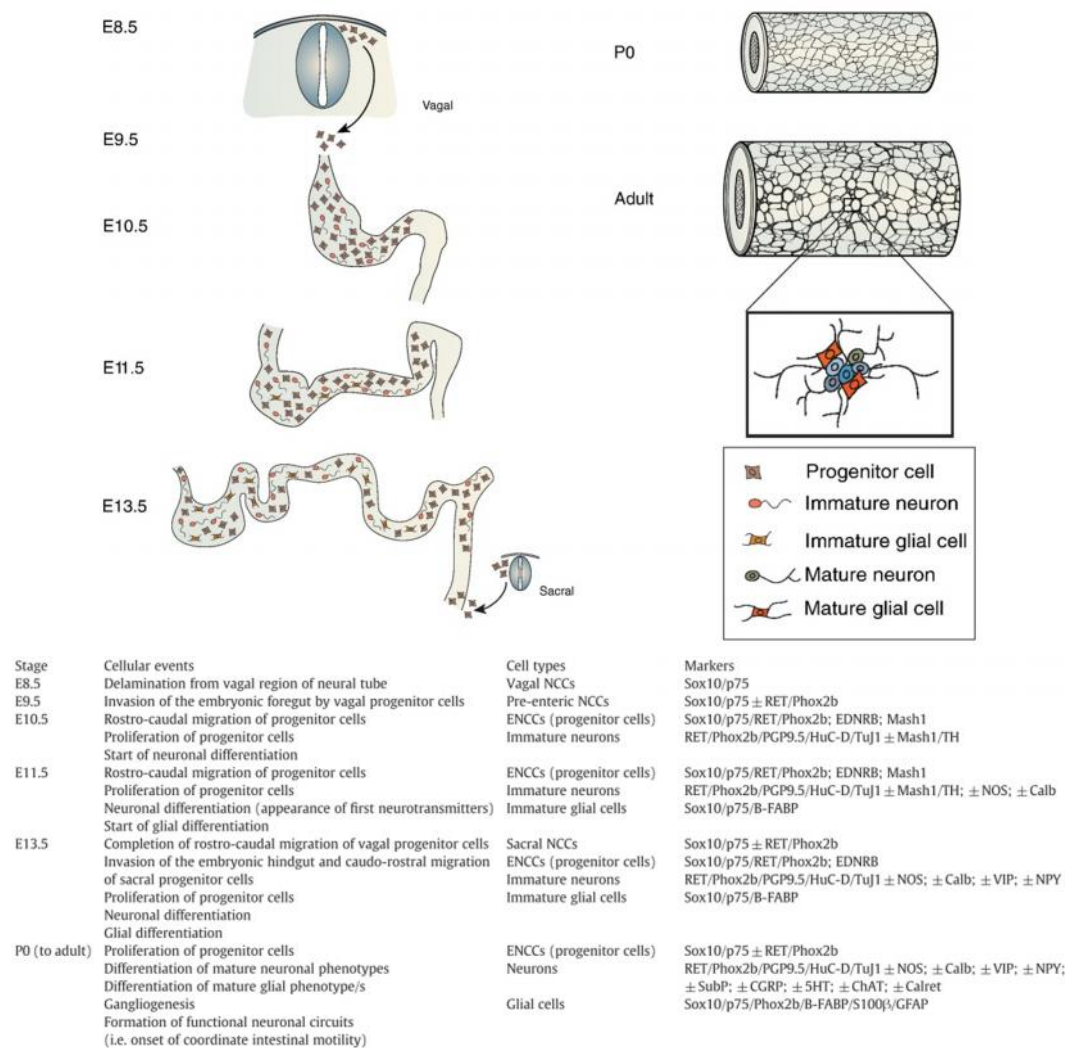| Stage	Cellular events	Cell types	Markers
E8.5	Delamination from vagal region of neural tube	Vagal NCCs	Sox10/p75
E9.5	Invasion of the embryonic foregut by vagal progenitor cells	Pre-enteric NCCs	Sox10/p75 ± RET/Phox2b
E10.5	Rostro-caudal migration of progenitor cells	ENCCs (progenitor cells)	Sox10/p75/RET/Phox2b; EDNRB; Mash1
	Proliferation of progenitor cells	Immature neurons	RET/Phox2b/PGP9.5/HuC-D/TuJ1 ± Mash1/TH
	Start of neuronal differentiation		
E11.5	Rostro-caudal migration of progenitor cells	ENCCs (progenitor cells)	Sox10/p75/RET/Phox2b; EDNRB; Mash1
	Proliferation of progenitor cells	Immature neurons	RET/Phox2b/PGP9.5/HuC-D/TuJ1 ± Mash1/TH; ± NOS; ± Calb
	Neuronal differentiation (appearance of first neurotransmitters)	Immature glial cells	Sox10/p75/B-FABP
	Start of glial differentiation		
E13.5	Completion of rostro-caudal migration of vagal progenitor cells	Sacral NCCs	Sox10/p75 ± RET/Phox2b
	Invasion of the embryonic hindgut and caudo-rostral migration of sacral progenitor cells	ENCCs (progenitor cells)	Sox10/p75/RET/Phox2b; EDNRB
		Immature neurons	RET/Phox2b/PGP9.5/HuC-D/TuJ1 ± NOS; ± Calb; ± VIP; ± NPY
	Proliferation of progenitor cells	Immature glial cells	Sox10/p75/B-FABP
	Neuronal differentiation		
	Glial differentiation		
P0 (to adult)	Proliferation of progenitor cells	ENCCs (progenitor cells)	Sox10/p75 ± RET/Phox2b
	Differentiation of mature neuronal phenotypes	Neurons	RET/Phox2b/PGP9.5/HuC-D/TuJ1 ± NOS; ± Calb; ± VIP; ± NPY; ± SubP; ± CGRP; ± 5HT; ± ChAT; ± Calret
	Differentiation of mature glial phenotype/s		
	Gangliogenesis	Glial cells	Sox10/p75/Phox2b/B-FABP/S100β/GFAP
	Formation of functional neuronal circuits (i.e. onset of coordinate intestinal motility)		

Figure 1.2. The development of the ENS in mice. Vagal neural crest cells migrate, proliferate and differentiate through the entire length of the developing gut. Sacral neural crest cells colonize the distal hindgut. The table summarizes the expression of different marker genes at different developmental stages. Adapted from [Sasseli et al., 2012].

Since the newborn ENS is not yet fully developed at birth an appropriate supply of neurotrophic factors is needed during the first postnatal weeks [Gershon et al., 1997; Schäfer et al., 1999]. Not all NCCs within the ENS

differentiate during development: a small pool of enteric neural crest stem cells persist in the postnatal and adult gut [Kruger et al., 2002]. Whether these cells could possess the same potential of NCCs is not clear: they are probably more restricted to a gut phenotype and more appropriately termed enteric neuronal precursors (ENPs). Several strategies have been developed in the last few decades to isolate and characterize ENPs due to their enormous therapeutic potential, regarding gastrointestinal motility or dysganglionic disorders (e.g. Hirschsprung´s disease) or even neurodegenerative diseases (e.g. Alzheimer´s and Parkinson´s disease). Moreover, the easy accessibility of this stem cell source, as well as the possibility of an autologous transplantation, make ENPs a feasible and valuable option for regenerative medicine. In fact, mucosal gut biopsies from human postnatal gut, obtained via minimally invasive endoscopic techniques, were demonstrated as a viable source of ENPs [Metzger et al.,2009; Rauch et al., 2006]. Such precursor cells have been isolated from the embryonic and postnatal gut of rats using antibodies to specific markers of enteric neural crest-derived cells such as RET, the tyrosine-kinase receptor for the glial cell-derived neurotrophic factor (GDNF) [Lo and Anderson, 1995], and the low-affinity nerve growth receptor p75 [Kruger et al., 2002]. In particular, Kruger and colleagues have isolated NCCs using flow cytometry sorting considering the expression of p75 and 4-integrin. The characteristic properties of neural stem cells, such as self-renewal and differentiative potential, were observed. In contrast, postnatal cells isolated using the same protocol revealed significant changes in self-renewal capacity and neuronal subtype potential (loss of serotonergic and noradrenergic potential) [Maslov et al., 2004]. ENPs isolated from the gut without using these markers and culturing with high concentrations of epithelial growth factor (EGF) and/or fibroblast growth factors (FGF), showed colonies called neurospheres, containing proliferating progenitor cells, neurons, and glia [Bondurand et al., 2003; Schafer et al., 2003; Suarez-Rodriguez and Belkind-Gerson, 2004; Metzger et al., 2009]. About 3-4% of the cells within neurospheres are actually true stem cells, able to generate all three neural lineages either *in vivo* or *in vitro* when induced to differentiate. Moreover, as in earlier studies also smooth muscle-like cells

were identified, it is still unclear if these cells arise from a common neural stem cell or from another, still unidentified myogenic progenitor [Kruger et al., 2002; Suarez-Rodriguez and Belkind-Gerson, 2004; Anitha et al., 2008; Metzger et al., 2009]. Nevertheless, the identification of the ENS stem cell niche, the involved signal pathways as well as cell-cell and cell-matrix interactions remains to be further characterized. The ENS has the ability to adapt itself to changing environmental cues under physiologically conditions; responding to diet, aging or injury throughout the entire lifespan of the organism. Several clinical and experimental observations suggest that gut homeostasis may be controlled by enteric ganglia [Thompson 1997]. Numerous "players" should be taken into account when considering adaptive changes in the enteric microenvironment (Figure 1.3): neurons, enteric glial cells, smooth muscle cells, interstitial cells of Cajal (ICCs) and immune cells. Moreover, putative mediators (neurotransmitters, growth factors, cytokines) are involved in the differentiation and adaptation of enteric neurons and glia.
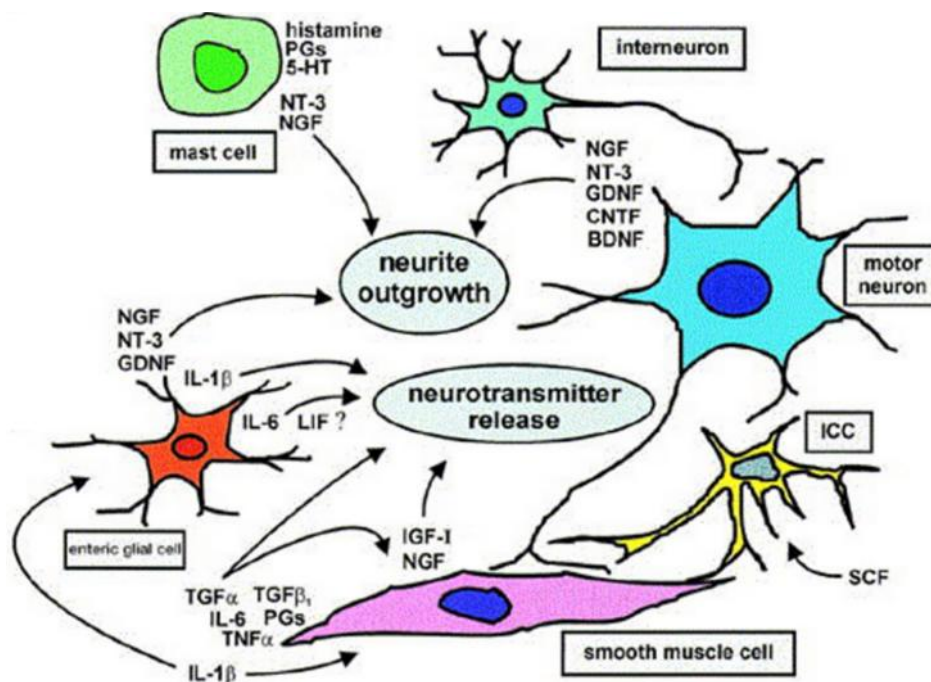

Figure 1.3. The enteric microenvironment. Schematic representation of different cell populations and putative mediators of their cross-talk. The interplay among different mediators is crucial to the regulation of ENS plasticity. Adapted from [Giaroni et al., 1999]

4

1.2 Cellular compartment

The main cell types constituting ENS are glial cells and neurons. The enteric glial cells are numerically larger than enteric neurons. They resemble to central nervous system (CNS) astrocytes and their cellular extensions cover the most of the enteric neuronal cell surface. The enteric glial cells produce interleukins and express MHC class II antigens in response to stimulation with specific cytokines [Cornet et al., 2001], suggesting a role of enteric glia in the modulation of gut inflammatory response. The enteric neurons can be divided into three types :

- *Type I*: innervating the smooth muscles , are highly excitable and are responsible for the nicotinic ganglionic synaptic transmission. Acting as motor neurons, they regulate the peristaltic gut.

- *Type II:* can be considered as sensory neurons receiving mechanical, thermal and chemical stimuli.

- *Type III*: similar to Type I, after being stimulated they generate postsynaptic excitatory potential.

However, ENS microenviroment is represented by other cell types such as smooth muscle cells, ICCs and immune cells.

Anatomically, ENS and, in particular, the myenteric plexus is embedded between the outer longitudinal and inner circular muscle layers. Recent evidences also suggest cross-talk between smooth muscle cells and dorsal root ganglion cells [Ennes et al., 1997]. Moreover, as previously described, an unique neural stem cell generating either neurons, glia and smooth muscle is hypothesized.

The ICCs lie in the plane of the myenteric plexus between the circular and longitudinal muscle layer. They are considered pacemaker cells controlling and modulating the electrical activity that give origin to the gut muscle contraction. These cells have been isolated and characterized by Sanders [1999] and colleagues using a surface glycoprotein CD34, functioning as a cell-cell adhesion factor. During differentiation ICCs co-express CD34 and the tyrosine-protein kinase Kit or CD117 [Maeda et al., 1992]. The expression of Kit has been used widely as marker of ICCs in a variety of

species [Sanders et al 1999], but its expression alone does not distinguish between the different classes of ICCs.

The gut-associated lymph tissue (GALT) contains about 70-80% of the body's immune cells and is the largest immune effector organ of the human body [Furness et al., 1999]. The GALT includes a high number of lymphocytes, specialized epithelial cells distributed on the surface, but also mast cells, macrophages and granulocytes. The lymphocytes, depending on their state of maturation, can be localized in the gut epithelium or in the *lamina propria*, influencing differently the response to neuropeptides and cytokines. Finally, mast cells may also play an important role in mediation of neuroimmune interactions [Buéno et al., 1997]

1.3 Soluble factors

Among soluble factors regulating the homeostasis of adult gut, important roles are played by GDNF, nerve growth factor (NGF), FGF, EGF, leukemia inhibitory factor (LIF) and Wnt proteins.

GDNF, a member of the TGF-b-superfamily [Lin et al., 1993], is a very important growth factor for the development and more importantly neuronal survival of the ENS. The lack of GDNF leads to a nearly complete loss of all enteric neurons [Moore et al., 1996]. *In vitro* studies of dissociated myenteric plexus from newborn rats have shown that GDNF improved neurite outgrowth and survival [Schäfer et al., 1999].

NGF has important developmental actions in both central and peripheral nervous systems. It has been demonstrated to promote neuroprotection [Friedman et al., 1999] and neuronal differentiation in primary cultures isolated from neonatal guinea pig myenteric plexus [Mulholland et al., 1994]. Loss or altered production of this factor are observed in enteric colitis correlating with postinflammatory irritable bowel syndrome (IBS) [Lin et al., 2005] and in aganglionic diseases such as Hirschsprung's [Kuroda et al., 1994].

Basic fibroblast growth factor (bFGF) is a mitogen for a variety of mesodermal and neuroectodermal derived cells [Gospodarowicz, 1986c]. It has been found in many tissues [Gospodarowicz et al., 1986a,b;

Gonzalez et al., 1990] and acts upon several types of cells [Baird and Klagsbrun, 1991]. The diverse source of bFGF raised the possibility that this neurotrophic factor also plays a role in the PNS. bFGF has been found in neurons and glia of the esophageal and colic myenteric plexus [Chadi et al., 2004]. Knockout model for bFGF showed specific neuronal cell loss in the ENS and abnormal development [Hagl et al., 2013]. These evidences suggest that this factor participates in the maintenance and plasticity of ENS.

Members of the EGF family growth factors are involved in mucosal protection and repair after injury and in tumor growth. Experimental evidences have demonstrated a protective effects of EGF-family members modulating epithelial cell migration [Dignass et al., 1993], mucosal blood flow [Hui et al., 1993], gastrointestinal motility [McLeay et al., 1990], mucous production and secretion [Kelly et al., 1990], and gastric acid secretion [Rhodes et al., 1986].

LIF has been shown to stimulate the generation of sensory neurons in cultures of mouse neural crest [Murphy et al. 1991]. Furthermore, LIF promotes the development of enteric neurons *in vitro*, particularly when it is given together with neurotrophin-3 (NT-3) [Chalazonitis et al. 1998]. These findings suggest a possible role for LIF in the development of the ENS. LIF also acts as a survival molecule in cultures of postnatal dorsal ganglion root ganglia [Murphy et al. 1991]. Deficiency of this factor has been reported in aganglionic bowel of Hirschsprung's patients suggesting an important role in survival of the enteric neurons [Wester and Olsen, 2000].

1.4 Wnt signaling in the gut

Wnt signaling has been implicated in the control of different types of stem cells, including hematopoietic [Reya et al., 2003] intestinal [Scoville et al., 2008] and neural stem cells in the CNS [Kléber et al., 2005; Ikeya et al., 1997] where it acts as a niche factor to maintain them in an undifferentiated and self-renewing state. The treatment of embryonic stem cells (ESCs) with an inhibitor of glycogen synthase-kinase-3 (GSK3) activates the canonical Wnt pathway and sustains pluripotency and self-

renewal, as indicated by the expression of pluripotency factors such as Oct-3/4 and Nanog [Sato et al., 2004]. Wnt signaling has already been proven to be essential for the development and migration of NCCs [Kléber et al., 2004; Stuhlmiller et al., 2012]. Furthermore, the presence of Wnt and its role in the development of the gut has been shown [Gregorieff et al., 2005; Theodosiou et al., 2003]. Wnt proteins bind specific surface receptors known as Frizzled. To date, 19 members of the Wnt family have been identified along with 10 members of the Frizzled family [Rao et al., 2010; Logan et al., 2004]. The binding of Wnt ligands to Frizzled receptors activates several distinct intracellular signaling pathways. These pathways include the Wnt/ -catenin (or canonical) pathway, the Wnt/Ca^{2+} pathway and the non-canonical pathway. The canonical pathway is activated by Wnt-1 class ligands (Wnt1/2/3/3a/8 and 8a), whereas the non-canonical pathways is activated by Wnt 5a class ligands (Wnt4/5a/5b/6/7a and 11) [Michaelidis et al., 2008]. This study has focused on the canonical pathway (Figure 1.4) which implies the presence of an extracellular Wnt1 class ligand, such as Wnt3a, interacting with one of its Frizzled receptors (FZD 1/3/9) and co-receptors for low density lipoproteins 5 and 6 (LRP5 or LRP6). This activation leads to the recruitment of the cytoplasmic components Dishevelled (Dvl) and Axin. Consequently, the key modulator of this pathway, GSK-3 , is inactivated by phosphorylation, thus resulting in accumulation of unphosphorylated -catenin that translocates into the nucleus. Inside the nucleus, -catenin forms complexes with different transcription factors, such as T-cell factor (TCF) and lymphocyte enhancer factor (LEF) families leading to the expression of Wnt target genes such as c-myc, cyclin and c-jun, which are involved in cell proliferation [Overview of -catenin target genes is showed on: http://www.stanford.edu/~rnusse/wntwindow.html]. In the absence of a Wnt signal, free cytoplasmic -catenin is recruited in a large "scaffolding" complex consisting of Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1) and the serine/threonine kinase GSK-3 . This cytoplasmic complex phosphorylates -catenin allowing the ubiquitination, mediated by -transducin repeat-containing protein (-TrCP), and subsequent -catenin cleavage by proteasome.
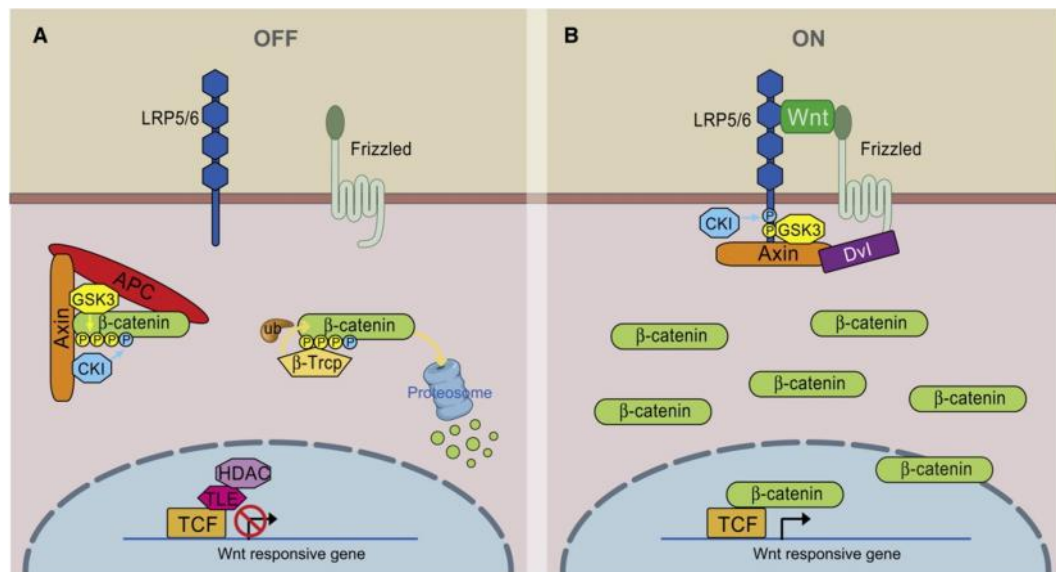
Figure 1.4. Wnt/ -catenin pathway (A) In the absence of a Wnt signal,cytoplasmic -catenin forms a complex with Axin, APC, GSK3 and CK1 and subsequentially GSK3. Following ubiquinition by -Trcp, phosphorylated -catenin is degradaded by the proteasome (B) When the Wnt ligand bind to its receptors Fz and LRP, Dvl binds to Fz leading to LRP phosphorylation and Axin recruitment. This disrupts Axin-mediated phosphorylation/degradation of -catenin, allowing -catenin to accumulate in the nucleus where it serves as a co-activator for TCF to activate Wnt responsive genes. Adapted from [MacDonald et al., 2010].

1.5 Influence of bacteria to ENS plasticity

The gastrointestinal microbiota consists of more than 10^{14} bacteria, which is 10 times the number of somatic cells in the human body [Luckey et al., 1972]. Under physiological conditions, the relationship between host and microbiota is a homeostatic symbiosis, in which the host provides the microbiota with nutrients and the microbiota contributes to the maintenance of the intestinal barrier and gut homeostastis Thus, the presence of commensal bacteria are important and have to be considered part of enteric microenviroment. In fact, lipopolysaccharides (LPS), an essential component of the outer membrane of gram-negative bacteria, have also been considered as important player in enteric plasticity; in conjunction with the previously described Wnt signals, LPS are key proteins involved in several processes such as proliferation and stem cell maintenance. LPS is released from luminal microflora and has been specifically linked to the Toll-like receptor 4 (TLR4) [Lien et al., 2000]. The LPS molecule consists of a polysaccharide, a core oligosaccharide, and a highly conserved lipid-A portion which triggers the inflammatory reaction

9

properties of LPS. Binding to TLR activates different intracellular signaling pathways ultimately leading to production of proinflammatory cytokines, chemokines and type 1 interferon [Kawai et al., 2006]. In pathological conditions, when these functions are deregulated, it may lead to inflammatory pathologies [Cario et al., 2005; Harris et al., 2006]. Inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis are chronic inflammatory disorders of the gastrointestinal tract (GIT) and occur as a result of complex interactions between the immune system and environmental factors such as luminal bacterial flora. Abnormalities in the structure of the ENS of IBD patients are frequently noted [Geboes et al., 1998; Villanacci et al., 2008], and gastrointestinal functions, such as motility and secretion that are controlled by the ENS have also been reported to be altered in IBD patients [Villanacci et al., 2008; Lomax et al., 2005; Vasina et al., 2006]. Recently, it has been shown that TLR4 is expressed on cells of the myenteric and submucosal plexus [Rumio et al., 2006; Barajon et al., 2009], highlighting the presence of a TLR-based direct neural response system to bacterial LPS in the ENS. Stimulation of myenteric neurons with LPS resulted in neuronal cell death [Arciszewski et al., 2005], whereas in another *in vitro* study, an increase in the proliferation of enteric glia has been observed [Cirillo et al., 2011]. LPS stimulation leads to intracellular activation of mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) that are responsible for the gene transcription of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), IL-1b, IL-12 and Interferon (IFN-γ) [Akira et al., 2004]. Previous studies have shown that LPS stimulation also acts on the phosphatidylinositol 3 kinase (PI3K) pathway, which is closely related to cell cycle regulation. In particular, it has been shown that LPS stimulation of TLR4 activates a cascade of PI3K leading to the phosphorylation of a protein kinase B (Akt), which in turn phosphorylates GSK-3β [Monick et al., 2001]. This protein, as described above, plays an important role in the Wnt canonical signaling. Similarly growth factors, such as EGF, bFGF2, GDNF, NGF and LIF, are also reported to interact with the β-catenin signaling pathway as demonstrated

in other studies [Ojeda et al., 2011; Srinivasan et al., 2005; Higuchi et al., 2003; Graf et al., 2011] (Figure 1.5).
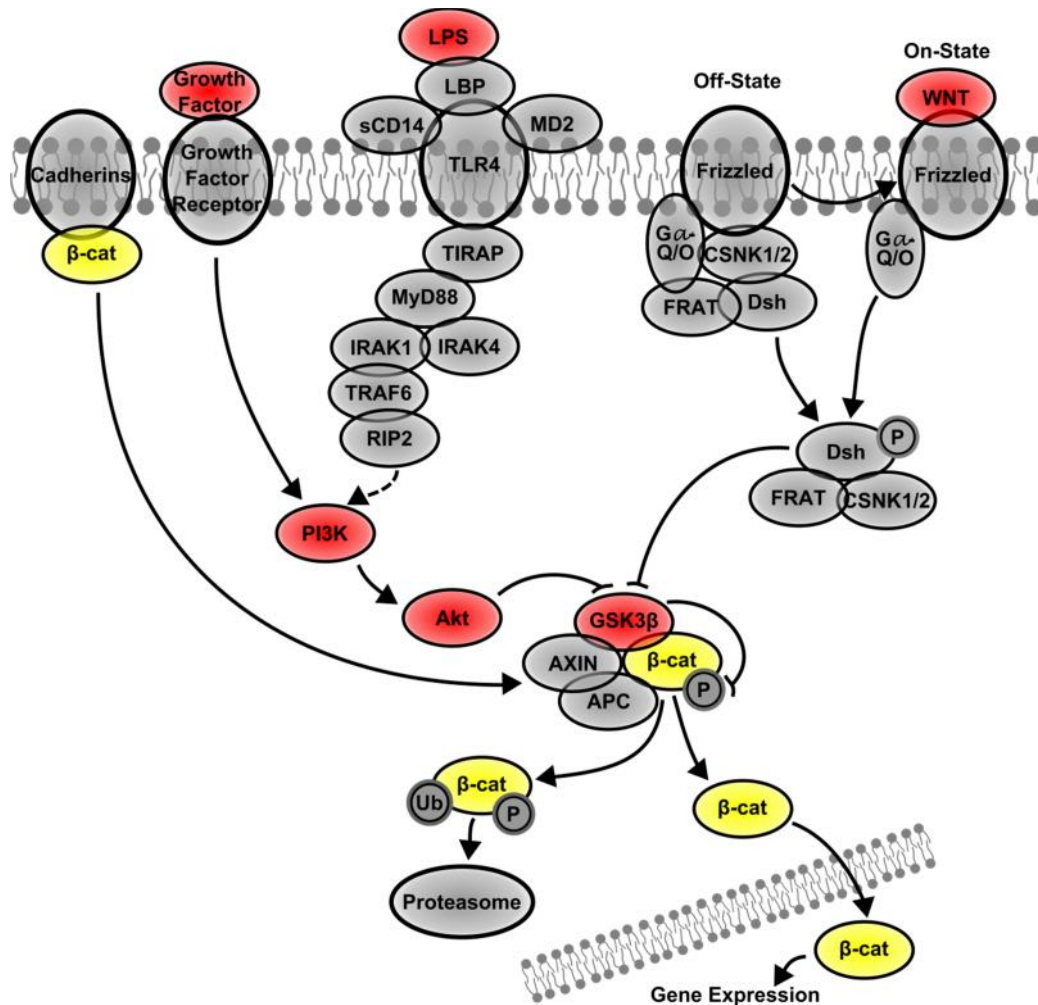

Figure 1.5. Schematic representation of hypothesized cross-talk between LPS/TLR4 and WNT/ -catenin signaling as well as involvement of growth factors.

1.6 Extracellular environment of ENS

Besides soluble factors, extracellular matrix (ECM) provide an important framework to the enteric microenvironment, during the enteric neural differentiation process, as well as the establishment and maintenance of the stem cell niche. The ECM is a network of different macromolecules mainly produced, secreted and assembled locally by cells, filling the extracellular spaces. The ECM is composed by a network of heteropolysaccharides, called glycosaminoglycans (GAGs), and fibrous proteins. GAGs are a family of polymers, consisting of disaccharide units, and divided into four groups according to their properties and chemical composition: hyaluronan, chondroitin, dermatan sulphate, heparan

sulphate and keratan sulphate. The last three groups differ from hyaluronan for two main aspects: they are generally shorter and covalently linked to specific proteins, thus forming proteoglycans. Proteoglycans are a heterogeneous group of molecules acting as tissue organizers, influencing the development of tissues and regulating the activity of secreted proteins. The major fibrous proteins present in the ECM are collagen, elastin, fibronectin and laminin, which form a complex network with proteoglycans giving strength and mechanical resistance to the matrix [Nelson D.L. and Cox M.M., Lehninger Principles of Biochemistry]. Among other functions, proteoglycans bind secreted proteins (e.g. FGFs and TGF-) and regulate their activity [Quarto et al., 1994]. ECM is implicated in the regulation of cellular phenotype contributing to maintain the distinct cellular environments, including the stem cell niches. There are at least three mechanisms by which the ECM can adjust the cell behavior. The first is through its composition, the second is due to synergistic interactions between growth factors and matrix molecules, and the third is mediated by cell receptors interacting with ECM components. These integrated proteins tether the cell to the ECM network and activate intracellular signaling pathways. The principal ECM receptors on cells, involved in cell adhesion, are integrins but also non-integrin receptors, like syndecan, CD36 and certain laminin binding proteins, such as CD133 [Rauch et al., 2003]. The ECM composition varies depending on the type of tissue and according to the different developmental stages. The ECM composition is not static: during development there are changes in the expression patterns of the individual components, demonstrating that ECM provides to cells specific microenvironmental information (Figure 1.6)
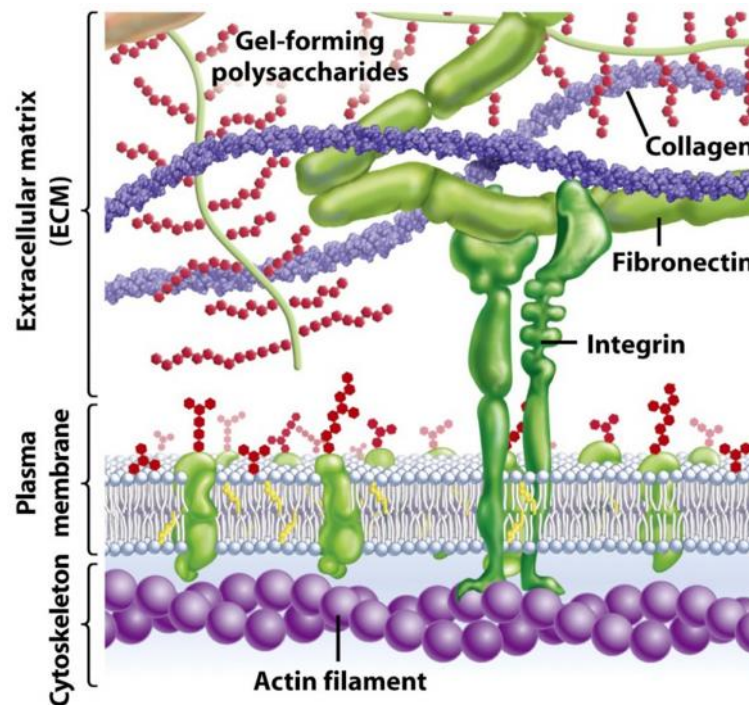
Figure 1.6. Schematic representation of ECM components interacting with cell protein such as integrins. [© Pearson Prentice Hall]

In the gut the ECM is composed principally by type I collagen, and by type III, IV, V and VI, fibronectin, laminin, decorin, biglycan, entactin, heparin, heparan sulfate and GAGs, including hyaluronic acid [Badilak et al., 2009]. During gut development, ECM molecules are necessary for NCC migration and cell differentiation. The cells respond to the heterogeneity of the ECM that forms their migration substrate. This continuous cell-cell and cell-matrix interactions are the main driving forces involved in morphogenesis and differentiation, not only during intestinal development but also in adult cell renewal [Simon-Assmann et al., 1995]. Fibronectin, for example, is expressed at high levels at sites of cell migration during ENS development. The role of laminin and other ECM components in cell migration has been shown in various cell culture studies [Adams et al., 1993] and also neurite outgrowth is supported by ECM components like laminin [Sanes et al., 1989], thrombospondin and vitronectin [Neugebauer et al., 1991]. Antibodies against 1 integrins blocked NCC adhesion to fibronectin, laminin and collagens, suggesting that these are the primary mediators of NCC attachment [Lallier et al., 1991; 1994]. ECM micro-environmental signals are necessary for the formation of enteric ganglia

[Pomeranz et al., 1993] and promote the ability of NCCs to give rise to neurons [Chalazonitis et al., 1997].

1.7 ECM application in tissue engineering

Tissue engineering is a multidisciplinary science that seeks to repair, maintain or enhance vital organs and tissues with diseases or dysfunctions [Langer et al., 1993]. The primary application of tissue engineering is in regenerative medicine. Preclinical and clinical studies have demonstrated that the use of biological materials, derived from extracellular matrices, facilitates the tissue remodelling. These biomaterials are organized in a three-dimensional structure containing tissue specific molecules [Badylak et al. , 2009]. The three-dimensional architecture of the biomaterial is essential and has to simulate *in vivo* situation. Microscopic and ultrastructural characteristics of the matrix modulate and control the cell migration and organization [Gong et al. , 2008; Sellaro et al. , 2007]. The biomaterials may be biological or synthetic matching essential ECM properties, such as biocompatibility, biodegradability, appropriate macro and micro structure, porosity, and chemical composition. The biological scaffolds do not contain synthetic materials and, depending on their origin, they may be prepared by chemical treatments. However, synthetic scaffolds are frequently composed by polymeric materials mimicking as much as possible the characteristics of the wild-type biological scaffolds. Biological scaffolds stimulate adhesion, proliferation and cell differentiation. Due to their origin, the high cost of production and the possible inflammatory responses as well as the difficulties to modulate the mechanical properties or the degradation, represent negative aspects. Synthetic scaffolds, obtainable by chemical synthesis, have low cost and controlled production, and it is possible to modulate the mechanical characteristics. However, inflammatory responses and often not adequate cell adhesion have been observed using these materials.

Decellularized or acellular matrices (AMs) have been used, as three-dimensional scaffold, to engineer several organs such as small intestinal submucosa [Badylak et al., 1989], heart [Ott et al., 2008], liver [Uygun et

al., 2010], esophagus [Macchiarini et al., 2008], and trachea [Conconi et al., 2005]. These organs are decellularized with different techniques that allow the preservation of the ECM ultrastructure. Depending on the type of tissue or organ, it is necessary to find a proper decellularization agent. The choice should take into account the type of tissue, the cell density, lipid content, and thickness [P. M Crapo et al. , 2011]. Decellularization agents may be chemical, biological, physical or mixed. An example of a chemical agent is given by the acids and bases that catalyze the hydrolytic degradation of biomolecules, or by hypotonic or hypertonic solutions which respectively cause cell osmotic lysis without changing the matrix structure [Xu et al., 2007; Cox et al., 2006]. Several protocols have used detergents, that may be ionic , non-ionic and zwitterionic. These chemical agents solubilize membranes and dissociate DNA from proteins leading to a removal of cellular material from the tissue [Cox et al., 2006; Giusti et al., 2009]. Finally, alcohols, such as glycerol, can be used to dehydrate and lyse the cells [Prasertsung et al., 2008]. With regard to the category of biological agents, enzymes, such as nucleases, trypsin, collagenase, lipase, dispase, thermolysin and -galactosidase have been used. Enzymes can provide high specificity to remove residual cells or specific proteic components of the ECM [Crapo et al. , 2011]. There are also non-enzymatic agents, such as ethylenediaminetetraacetic acid (EDTA) and etilenglicoletetracetic acid (EGTA), which help the cell dissociation from ECM proteins by binding metal ions [Klebe et al., 1974; Gailit et al., 1988]. Instead, the physical agents include temperature, force and pressure. The processes of freezing and thawing lead to cell lysis whereas membranes and the remaining cell contents must be removed by other agents.

1.8 *In vitro* models of ENS

Developmental processes, differentiation and plasticity studies using 2-dimensional cell cultures cannot properly mimic the *in vivo* situation. Given the importance of the ECM in these processes, new three-dimensional *in vitro* models need to be developed. Schäfer and colleagues [2000] have shown that culturing ENS cells in an ECM gel leads to the development of an enteric neurons and glial network similarly to *in vivo* situation. ECM gel,

supplemented with collagen II, has been used to established a three-dimensional co-culture model, evaluating the interactions between the enteric neural compartment and enterocytes [Holland-Cunz et al., 2004]. Intestinal AM could serve as a three-dimensional *in vitro* model to study several cellular processes since it mirrors the composition and structure of the actual organ.

2. Aims of the study

This study was focalized to further characterize the ENS cell population isolated from the small intestine of neonatal rats and evaluate the influences of microenviromental stimuli such as: soluble factors, cell-cell interaction and extracellular matrix. *In vivo* conditions which provide a reduction or absence of these factors promote the development of diseases such as intestinal agangliosis. Thus, two models of *in vitro* culture that simulate the physiological condition (SM) and that of agangliosis (BM) was considered. Particular attention was given to ENPs within the ENS and the involvement of specific pathways such as the WNT/ß-catenin dependent signaling. Moreover, due to the continual presence of commensal and pathological bacteria within the gut, the interaction between LPS/TLR4 and Wnt signaling was investigated. Finally, extracellular matrix contribute to maintenance of ENS homeostasis and differentiation was evaluated assessing an *in vitro* model represented by ENS-derived cells cultured on a decellularized ECM of adult rat jejunum.

3. Materials and Methods

3.1 Isolation, culture and stimulation of ENS-derived cells

All animal procedures were performed under the guidelines of the local ethic committee and according to Italian laws for animal protection. Sprague Dawley rats (3 days old, P3), were sacrificed by decapitation. Briefly, the smooth muscle layer was stripped from the mucous one and incubated in a digestion medium at 37°C, according to previously described protocols [Schäfer et al., 1997]. The digestion medium was composed of Hank's balanced salt solution (PAN, Aidenbach, Germany), 50 ng/ml trypsin-chymotrypsin inhibitor (Sigma-Aldrich, Milan, Italy), 1 mg/ml collagenase type 2 (Worthington, Lakewood, USA) and 200 µg/ml deoxyribonuclease (DNAse) (Roche, Mannheim, Germany). Myenteric nets were collected and treated with accutase (PAA, Pasching, Austria) for 10 min. After the digestion, cells were dissociated by aspiration through a 27G needle. The preparation yield per animal was approximately 1×10^6 cells. The cells were seeded at a density of 2×10^5 cells/cm². The isolated cells were either cultured in standard conditions [Schaefer et al., 1997] or under basal conditions for 7 days before morphological analysis by optical microscopy, immunophenotypical characterization by flow cytometry (FCM) and Wnt signalling study by transfection, gene expression, western blotting (WB) and immunofluorescence (IF).

Basal conditions (BM): The basal medium was composed of Neuronal Base P (PAA) supplemented with 1% L-glutamin (Sigma-Aldrich) and 1% of penicillin/streptomycin (Invitrogen, Milan, Italy).

Standard conditions (SM): The complete medium contained Neuronal Base P (PAA) supplemented with 2% neuronal stem cell supplement (PAA), 1% bovine serum albumin (Sigma-Aldrich), 0.1% -mercaptoethanol (Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 1% L-glutamine (Sigma-Aldrich), 10 ng/ml EGF (ImmunoTools, Friesoythe, Germany), 20 ng/ml b-FGF (ImmunoTools) and 10 ng/ml GDNF (ImmunoTools).

3.2 Immunophenotypical characterization of primary cultures by Flow Cytometry (FCM)

ENS cells were characterized at different time points (T0 and T7 days) using the primary antibodies reported in Table 1.

Primary antibody	Manufacture's company
Mouse anti-rat NG2 FITC	Santa Cruz
Goat anti-rat Nanog-PE	BD Bioscience
Mouse anti-rat CD34 PE-Cy7	BD Biosciences
Rabbit anti-rat Sox2	Millipore
Rabbit anti-rat Sox10	Santa Cruz
Rabbit anti-rat TLR4	Santa Cruz
Goat anti-rat Frizzled 1	Santa Cruz
Goat anti-rat Frizzled 3	Santa Cruz
Goat anti-rat Frizzled 9	Santa Cruz
Goat anti-rat Nestin	Santa Cruz
Mouse anti-rat GFAP	Millipore
Mouse anti-rat Pan neuronal antigen	Millipore
Rabbit anti-rat p75	Millipore
Mouse anti-rat Tuj-1	Millipore

Table 1. Primary and secondary antibodies used for the characterisation of ENS cells by FCM

For indirect staining, FITC- and PE- conjugated secondary antibodies (Santa Cruz, Dallas, USA) were considered. Samples labelled with isotypic or secondary conjugated antibodies were prepared as negative controls. Data were acquired using flow cytometer FACSCanto II (BD Biosciences, Milan, Italy) and then analyzed with the *Substruction* tool of Summit 4.3 software (DAKO-Beckman Coulter).

3.3 Wnt signalling analysis

A. Transfection

In order to study the activation of Wnt canonical pathway, a cell transfection procedure was performed using Roti®-Fect PLUS delivery system (Carl ROTH, Karlsruhe, Germany) and GFP Wnt reporter plasmid (Top_EGFP3a), kindly provided by Prof. Oliver Müller (University of Applied Sciences, Kaiserslautern, Germany) and designed with a LEF1 motif linked to an inducible promoter upstream of GFP gene (Figure 3.1). Sub-confluent (70-80%) ENS cells were transfected with 2 µg of DNA- Top_EGFP3a (Top cells), while GFP Reporter Plasmid (EGFP) and empty vector were used to prepare positive and negative transfection controls, respectively.   The transfection efficiency was determined detecting GFP positive cells by FCM and optical fluorescence microscopy (Cell Observer Z1, Zeiss Germany).
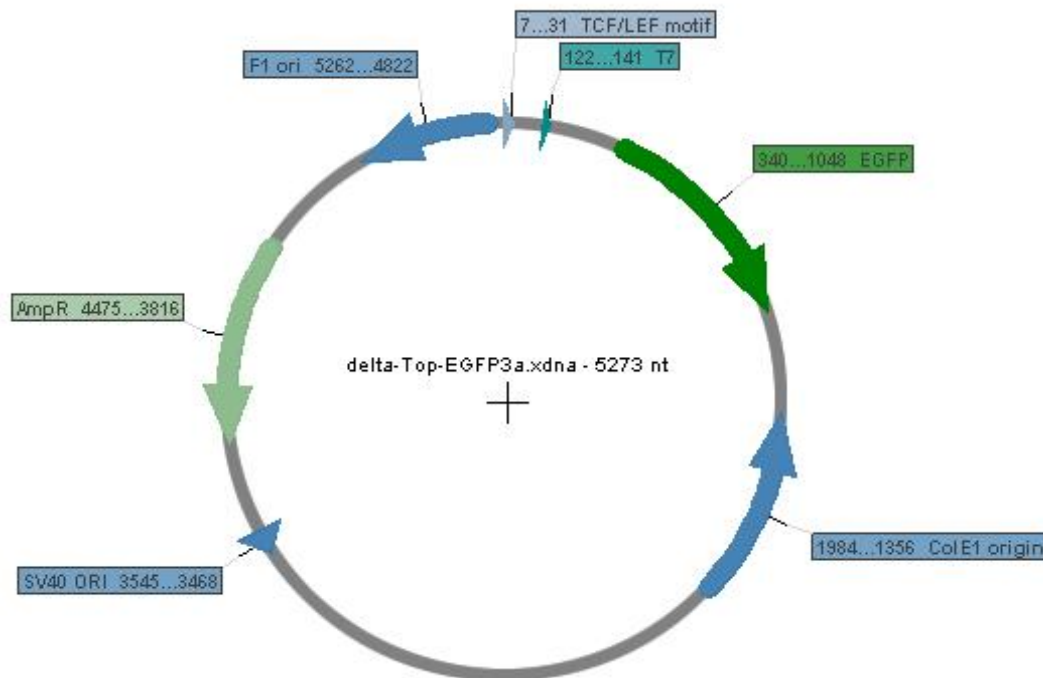

Figure 3.1 . Delta-Top-EGFP3a plasmid design scheme.

B. Stimulation

After 24h, transfected cells were stimulated with 20 ng/mL rhWnt3a (R&D system, Wiesbaden-Nordenstadt Germany). In order to define the correlation between Wnt signalling and LPS/TLR4 pathway, Top cells

were stimulated with 5 µg/mL LPS (Sigma-Aldrich) and then studied for GFP expression and cytoplasmic -Catenin accumulation by FCM.

C. -Catenin nuclear translocation

At different time points (T24h-T96h), the transfected cells were fixed with BD Cytofix solution (BD Biosciences) for 20 min, at 4°C. For intracellular antigens, permeabilization and aspecific site blocking step were performed using 0.5% Triton (Sigma-Aldrich) and 1% BSA solution, respectively. All samples were then double stained with primary mouse anti-rat -Catenin (Santa Cruz) –PE indirectly conjugated and mouse anti-rat LEF1-PE Cy7 conjugated (Cell Signaling, Danvers, USA) antibodies. As negative controls, specimens stained only with secondary PE-conjugated and isotypic antibodies were prepared. After mounting with Fluoro-gel II solution containing DAPI (EMS, Hatfield, USA), slices were observed using a Leica SP5 TCS confocal microscope (Leica, Wetzlar, Germany).

D. Interaction of Wnt signalling with LPS pathway

As previous studies reported a molecular intersection between components of the Wnt and PI3K/Akt signaling pathways in enteric epithelial cells [Scoville et al, 2008], we investigated the downstream crosstalk of LPS and Wnt3a stimulus in ENS-derived cells treated with 5 µg/mL LPS or 20 ng/mL rhWnt3a under standard (SM) or basal conditions (BM) for 24h. At different intervals (0.5-12, 24h), nuclear and cytoplasmic proteins were extracted using NER-PER Nuclear and Cytoplasmic Extraction Reagents kit and quantified by BCA Protein Assay Reagent Kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. Proteins were separated by reducing sodium dodecyl sulfate/ polyacrylamide gel electrophoresis (SDS⁄PAGE) (BioRad, Milan, Italy) and then electrophoretically transferred onto polyvinylidene difluoride (PVDF) (Millipore, Billerica, USA) by overnight incubation at 4°C with rabbit anti-rat p-GSK-3 , mouse anti-rat-pAkt (Cell signaling), - -Catenin, -p- -Catenin and rabbit-anti rat NF- B (Santa Cruz) primary antibodies. After washing with phosphate buffer (PBS) containing 0.25% Tween-20, the membranes

were incubated for 1h with peroxidase-conjugated secondary goat anti-rabbit and goat anti-mouse antibodies (BioRad) and then developed using an enhanced chemiluminescence substrate (ECL) (Sigma-Aldrich). Immunoreactivity was visualized by VersaDoc Imaging System (BioRad) and protein expression was normalized to housekeeping protein GAPDH (Millipore) and lamin B (Santa Cruz) for cytoplasm or nuclear extract, respectively. Quantification of protein expression was performed using the image processing software ImageJ. Data were reported as ratio within target protein and relative housekeeping protein expression. Statistical significance, calculated by t-Test, compared to control: p-value 0.05: *, p-value 0.01: ** .

3.4 Co-immunoprecipitation: Wnt3a and Frizzled-9 binding assay

In order to determine the interaction between Frizzled-9 and Wnt3a, total protein extraction was performed on ENS cells cultured for 7 days using RIPA lysis buffer. Recombinant Wnt3a protein (R&D System) was added (200 ng/mL) to total cells protein extracts and incubated overnight at 4° C. Immunoaffinity purification was performed using goat anti-rat Frizzled 9 (Santa Cruz) and rabbit anti-rat Wnt3a (Cell signaling) antibodies immobilized onto Protein A-Sepharose (Sigma-Aldrich). Western blot analysis was carried out using 4-15% Mini-PROTEAN® TGXTM Precast Gel (BioRad) and goat anti-rat Frizzled 9 (Santa Cruz) and rabbit anti-rat Wnt3a (Cell signaling) antibodies were used for immunoblot detection.

3.5 Immunofluorescence

ENS cells cultured for 7 days with BM or SM were fixed with BD Cytofix solution (BD Biosciences) for 20 min, at 4°C. All samples were double stained with primary goat anti-rat Frizzled-9, rabbit ant-rat TLR4 (Santa Cruz) and rabbit ant-rat Wnt3a (Cell Signalling) antibodies. Immunodetection was evaluated indirectly with –PE and –FITC conjugated antibodies respectively. As negative controls, specimens stained only with secondary antibodies were prepared. After mounting with Fluoro-gel II

solution containing DAPI (EMS, Hatfield, USA), slices were observed using a Leica SP5 TCS confocal microscope (Leica, Wetzlar, Germany).

3.6 Gene expression study

At 24h, 72h and 7 days from stimulation with rhWnt3a and LPS, total cellular RNA was extracted using TRIzol® (Invitrogen), quantified by measuring the absorbance at 260 nm and stored at -80 °C until use. RNA (10 ng) was reverse transcribed and amplified in a iCycler iQ ™ (BioRad Laboratories, Hercules, CA, USA), using a Qiagen One Step RT–PCR Kit (Venlo). Primer pairs for target genes and the housekeeping gene Hypoxanthine phosphoribosyltransferase (HPRT) were designed (Table 2) and purchased from Invitrogen Life Technology. RT–PCR products were electrophoresed on a 2% agarose gel (Invitrogen) and visualized using a UV-transilluminator Gel Doc 2000 Gel Documentation System (BioRad).

Gene	Abbreviations	Primers sequences	Accession
Glial cell-derived neurotrophic factor	GDNF	F: CCAGAGAATTCCAGAGGGAAAG R: CTTCACAGGAACCGCTACAA	NM_019139.1
Epidermal growth factor	EGF	F: GGGCTATCCCATCGGTAATAAG R: CAGCCTCCATTCCTGTGTAA	NM_012842.1
Basic fibroblast growth factor	FGF2	F: ACAGGAGTTGTGTCCATCAAG R: CTCCAGGCGTTCAAAGAAGA	NM_019305.2
Nerve growth factor	NGF	F: CAGTGTGTGGGTTGGAGATAA R: GCATCCACTCTCTACAGGATTC	NM_001277055.1
Leukemia inhibitory factor	LIF	F: TGACGGATTTCCCACCTTTC R: CGTCTGTAGTCGCATTGAGTT	NM_022196.2
Hypoxanthine-guanine phosphoribosyltransferase	HPRT	F: GCTGACCTGCTGGATTACAT R: CCCGTTGACTGGTCATTACA	NM_012583.2

Table 2. Oligonucleotides used for the One Step RT-PCR analysis (F=Forward; R=Reverse).

For genes reported in Table 3, the analysis of gene expression was conducted by qPCR. In particular, the reverse transcription was done using Thermoscript ™ RT-PCR System kit (Invitrogen) and the amplification reaction was performed using Platinum ® SYBR ® Green qPCR SuperMix-UDG kit (Invitrogen) and DNA Engine Opticon ® Real-Time Thermal Cycler (MJ Research, St. Bruno, Canada). Amounts of each gene product were calculated using linear regression analysis from standard curves, demonstrating amplification efficiencies ranging from 90 to 100%. The term "fold induction" was defined as the cDNA ratio between target gene and reference gene (HPRT) normalized to untreated control. Statistical significance, calculated by t-Test, compared to untreated

samples: p-value   0.05: *, p-value   0.01: **; compared to LPS treated cells: p-value   0.01:   .

Gene	Abbreviations	Primers sequences	Accession
Toll-like receptor 4	TLR4	F: ATTGCTCAGACATGGCAGTTTC R: CACTCGAGCTAGCTCTTTCTCCTAA	NM_019178.1
Wingless-type MMTV integration site family, member 3A	WNT3A	F: TGCAAATGCCACGGACTATC R: AGACTCTCGGTGTTTCTCTACC	NM_001107005.2
Frizzled 9	FZD9	F: TACCCAGAGCGCCCTATAAT R: CAAACCCTCCTCCATCACATAC	NM_153305.1
Axis inhibition protein 2	AXIN-2	F: AGCTATGCCTGTCTCCTCTAAG R: GTCCAGGGTATCCACACATTC	NM_024355.1
Myelocytomatosis proto-oncogene	CMYC	F: CTTGGAACGTCAGAGGAGAAA R: GCTTGAACGGACAGGATGTA	NM_012603.2
Jun proto-oncogene	C-JUN	F: GAAGCAGAGCATGACCTTGA R: CCATTGCTGGACTGGATGAT	NM_021835.3
Interleukin -1β	IL-1β	F: AGTGAGGAGAATGACCTGTTC R: CGAGATGCTGCTGTGTGAGATT	NM_031512.2
Interleukin-6	IL-6	F: GCCACACTCATTCACACCAATA R: GTTGGATGGTCTTGGTCCTTAG	NM_26744.1
Interleukin-10	IL-10	F: ATTCAACCACCCCGGCATCTA R: CAACCAGCTTTTCCAACGAG	NM_012854
Tumor necrosis factor α	TNFα	F: GCAGATGGGCTGTACCTTATC R: GGCTGACTTTCTCCTGGTATG	NM_012675.3
Chemokine (C-C motif) ligand 3	CCL3	F: TCTCCTATCCACGCCAAATTC R: TCTTGCACCCAGCTCTCTT	NM_013025.2
Hypoxanthine-guanine phosphoribosyltransferase	HPRT	F: GCTGACCTGCTGGATTACAT R: CCCGTTGACTGGTCATTACA	NM_012583.2

Table 3. Oligonucleotides used for the Real-Time PCR analysis (F=Forward; R=Reverse).

3.7 Preparation of gut acellular matrix

The decellularization protocol for adult rat jejunum was adapted from previous decellularization protocols of tracheas [Conconi et al., 2005]. Briefly, the adult rat was sacrified by cervical dislocation and the intestine was removed and rinsed several times with PBS and Amuchina (Angelini, Roma, Italy). Subsequently, the luminal surface was treated with 2U/ml Dispase II (Roche) at 37°C for 1h. Afterwards, the tissue underwent decellularization cycle composed of: steriled Milli-Q water supplemented 1% of antibiotic solution for 72h, 4% sodium deoxycholate (Sigma-Aldrich) for 4h, and 2000 kU DNase-I (Sigma-Aldrich) in 1 M NaCl (Sigma-Aldrich) for 3 h. Cycle was repeated until the tissue was completely decellularized (Figure 3.2). The absence of cellular elements was verified by Toluidine blu staining, DAPI staining and scanning electron microscopy (SEM). Acellular matrices (AMs) were stored in PBS supplemented with 1% antibiotic solution at 4°C until use. Untreated jejunum served as a control.
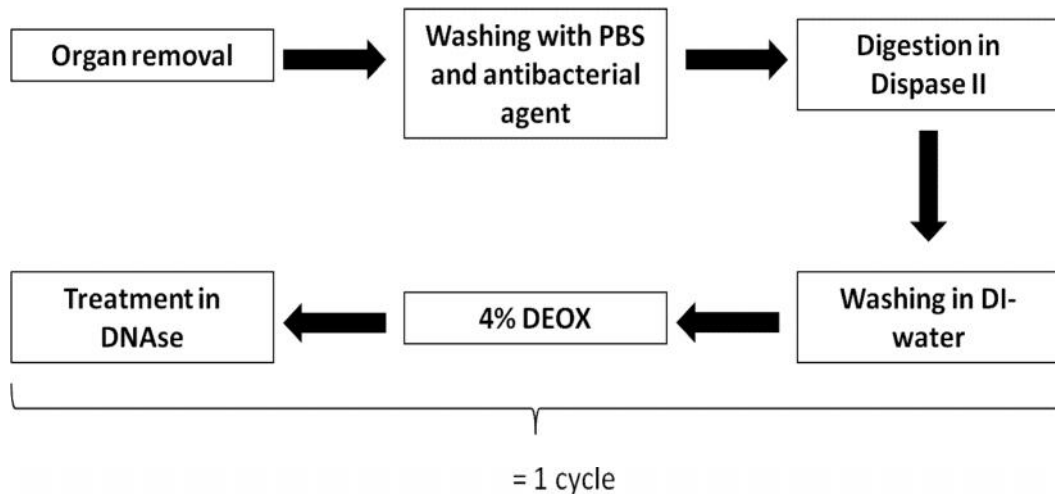
Figure 3.2. Schematic diagram representing the decellularization process

3.8 Morphological and protein characterization of AM

A. Histochemistry

AMs were opened and pieces of about 5 mm^2 were embedded in cryostat embedding medium (Killik) (Bio-Optica, Milano, Italy). Then, the samples were cut (7 µm) using Cryostat DM2000 (Leika,) and put onto microscope slides (Thermoscientific). After fixation with cold acetic acid for 5 min, toluidine blue staining was performed and sections were mounted with Pertex solution (Leika). In parallel, after fixation cryosections were mounted in Fluoro-gel II containing DAPI (EMS).

B. SEM

Surface morphology was evaluated by SEM. After fixing, the samples were dehydrated through a graded series of ethanol-water mixtures from 10% to 100% ethanol. The samples were kept in absolute alcohol until analysis and then subjected to Critical Point Drying and metalized with gold. The images were acquired using the scanning electron microscope (JSM 6490) (JEOL, Peabody, USA).

C. Immunofluorescence

The AM were characterized for the presence of specific ECM proteins. Cryosections (7 µM) were permeabilized with Triton X-100 (Sigma) for 30

min and nonspecific sites were blocked with 1% BSA (Sigma) in PBS. AMs were stained with rabbit anti-rat -laminin, - collagene I and -collagene III (CosmoBio, Tokyo, Japan) antibodies and incubated overnight at RT. Thenafter, the sections were treated for 1h at RT with secondary antibody goat anti-rabbit-FITC (Santa Cruz). AM sections treated with only secondary antibody were considered as negative control. Samples were mounted with Fluoro-gel II (EMS) and examined using a fluorescence microscope (Leica SP2).

D. Quantification of DNA and RNA from AM

AM samples (5 mm ² and 0,020g), after III, IV and V cycles, were processed for DNA and RNA extraction using Trizol® Reagent (Sigma-Aldrich) following manufacture's protocol. The untreated sample was considered as positive control. Quantification was performed using the NanoDrop 2000 spectrophotometer (Thermo Scientific).

E. Western blot

Collagene I and IV expression were evaluated by western blotting following the protocol described previously. Briefly, total proteins extracted from AMs (20 µg) were separated by SDS/PAGE and electrophoretically transferred onto PVDF membranes. Rabbit anti-rat –collagene I and –collagene IV (CosmoBio) antibodies were used for immunoblot detection. Immunoreactivity was visualized by VersaDoc Imaging System (BioRad).

3.9 *In vitro* cultures of ENS cells on AM

Primary ENS cells, isolated from postnatal (P3) rat guts were seeded on top of the outer layer of decellularized jejunum, either directly after isolation (T0) or after seven days of culture (T7) in BM or SM. The decellularized tissue was opened and cut into pieces of 5 mm^2. The matrix was stretched on sterile coverslip glass, transferred into 24 well plates (BD, Falcon) and fastened with metal rings. Cultures were fixed after different time points (3, 7, 14 days) with 4% glutaraldehyde for SEM analysis or with Cytofix solution (BD Biosciences) for wholemount staining.

A. Whole mount immunofluorescence staining

For wholemount staining, the specimens were fixed in Cytofix (BD Bioscience) for 2h and permeabilized with 0.5% triton X-100 (Sigma-Aldrich) for 30 min following a blocking step with 1% BSA (Sigma-Aldrich) for 2h at 4°C. Primary antibodies against ß-III tubulin (Millipore) and -sma-FITC (Abcam, Cambridge, UK) were incubated overnight at RT. Visualization resulted from a secondary antibody anti-mouse PE (Santa Cruz) which was incubated for 3h at RT. Samples were mounted with Fluoro-gel II containing DAPI (EMS) and examined using a fluorescence microscope (Leica SP2).

B. Gene expression

After 3, 7 and 14 days of cultures, repopulated AMs were treated with TRIzol® (Invitrogen Life Technology, Carlsbad, CA, USA), total RNA was quantified by measuring the absorbance at 260 nm and stored at -80 °C until use. RNA (30 ng) was reverse transcribed and amplified in a iCycler iQ ™ (BioRad Laboratories, Hercules, CA, USA), using a Qiagen One Step RT–PCR Kit (Venlo). Primer pairs for target genes and the housekeeping gene Hypoxanthine phosphoribosyltransferase (HPRT) were designed (Table 4) and purchased from Invitrogen Life Technology. RT–PCR products were electrophoresed on a 2% agarose gel (Invitrogen) and visualized using a UV-transilluminator Gel Doc 2000 Gel Documentation System (BioRad).

4. Results and Discussion

4.1 ENS-derived cells cultured in basal condition does not generate neurospheres.

It is known that growth factors, such as EGF, bFGF and GDNF play an essential role in the development and homeostasis of the ENS. *In vivo* conditions which provide a reduction or absence of these factors promote the development of diseases such as intestinal agangliosis. Thus, two models of *in vitro* culture that simulate the physiological condition (SM) and that of agangliosis (BM) were evaluated. After 7 days of culture in SM, typical neurosphere formation with some adherent cells was visible, whereas no neurospheres were observed in BM. However, the culture showed several colonies resembling fibroblastic colon-forming units (CFU-F) (Figure 4.1)
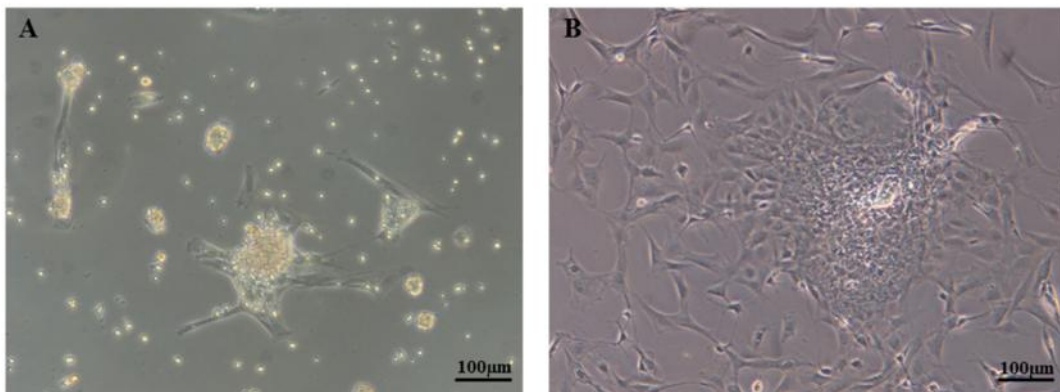

Figure 4.1. Phase contrast microscopy of ENS-derived cells cultured for 7 days in SM (A) and BM (B).

4.2 Basal culture condition stimulates Frizzled 9-expressing cells against TLR4 positive subpopulations

As demonstrated by flow cytometrical analysis, primary ENS cultures were heterogeneous presenting characteristic stemness grade (Figure 4.2), as suggested at T0 by the expression of Nanog (26.9 ± 0.6%), SOX2 (64.9 ±1.4%), SOX10 (17.7 ± 0,4%) and p75 (2.2 ± 0.4%). Multidifferentiative potential of freshly isolated rat ENS cells was confirmed by the expression of

chondroitin sulphate NG2 (51.9 ± 4.3%), a proteoglycan tipically observed on the membrane of pericyte-like cells [Corselli et al., 2010], circulating multipotent blood-derived cells [Traktuev et al., 2008], specific glial cells [Richardson et al., 2001; Trotter et al., 2011] and multipotent neural stem cells [Nishiyama et al., 2009]. Moreover, the presence of Nestin (24.0 ± 3.3%) and CD34 (35.9 ± 1.2%) was correlated to the presence of neural precursor cells [Belkind-Gerson et al., 2013], ICCs [Sanders et al., 1999] as well as stem-like cells [Holyoake et al., 1994; Asahara et al., 1997; Zvaifler et al., 2000], respectively. Frizzled 9, present in brain and neural crest, was expressed by 17.5 ± 3.0%, while Frizzled 1 and 3, that are known to be expressed in central nervous system [Wang et al., 1997; Lee et al., 2012; Deardorff et al., 2001] were not significantly detected at T0. The presence of differentiated glial cells was revealed by the expression of GFAP (22.5 ± 0.6%), whereas the responsiveness of ENS populations to LPS stimulus was suggested by TLR4 receptor (19.5 ± 0.7%).
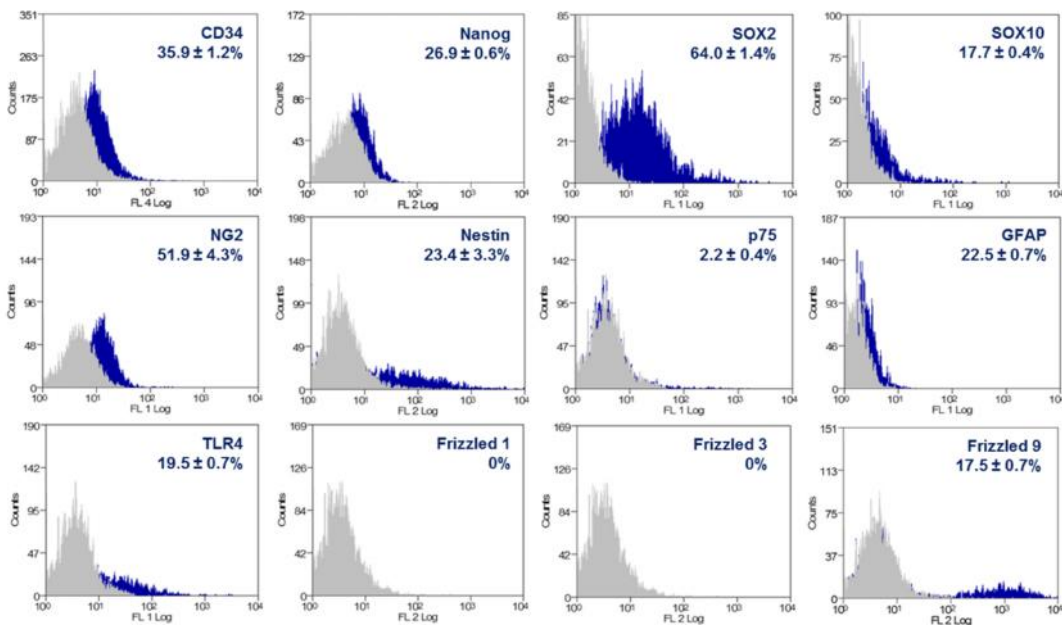

Figure 4.2. FCM analysis of rat ENS cells after 7 days in either SM or BM. Data are expressed as percentage ± SD of positive cells (blue profile) with respect to controls (grey profiles) prepared using isotopic or secondary antibodies.

After 7 days of culturing, a different immunophenotypical expression was observed in cultures treated with standard and basal medium (Figure 4.3).
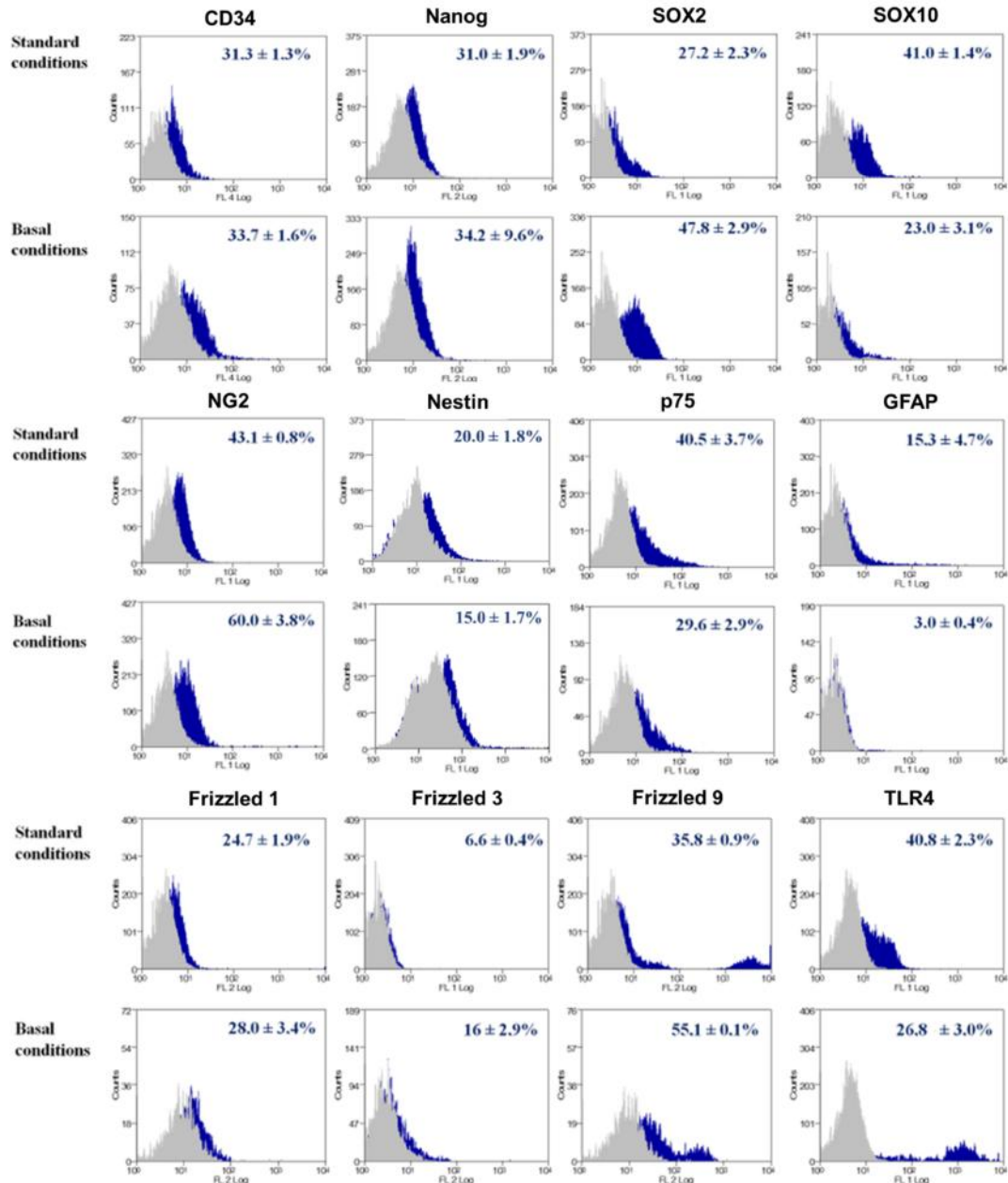

Figure 4.3. Flow cytometry analysis of rat (P3) ENS cells after 7 days in either SM or BM. Data are expressed as percentage ± SD of positive cells (blue profile) with respect to controls (grey profiles) prepared using isotopic or secondary antibodies.

As previously reported [Graham et al., 2003], SOX2 showed to be downregulated in cells cultured in SM (27.2 ± 2.3%) and in BM (47.8 ± 2.9%). In contrast, a significant increase of positivity for the pluripotency marker

Nanog (31.0 ± 1.9% in SM; 34.2 ± 9.6% in BM) and SOX10 (41.0 ± 1.4% in SM; 23.0 ± 3.1% in BM) was detected. SOX2, a transcription factor important for the maintenance of self-renewal [Heanue et al., 2011], was reduced whereas SOX10, considered also as a glial committed cell marker [Britsch, 2001], increased. The significantive higher reduction and increase of SOX2 and SOX10, respectively, in SM in comparison with BM, demonstrate that factors present in the culture medium induce differentiation. Moreover, ENS-derived cells showed to be enriched of cells positive for p75 (40.5 ± 3.7% in SM; 29.6 ± 2.9% in BM), Frizzled 1 (24.7 ±1.9% in SM; 28.0 ± 3.4% in BM), Frizzled 3 (6.6 ± 0.4% in SM; 16.0± 2.9% in BM) and Frizzled 9 (35.8 ± 0.9% in SM; 55.1 ± 0.1% in BM). Frizzled 3 is reported to be involved in neural crest development [Deardorff et al., 2001] and Frizzled 9 is expressed on neural precursors [Van Raay et al., 2001]. Frizzled 1 is known to be localized in the neural stem cells niche of subventricolar zone (SVZ) and interact with Wnt3a [Lee et al., 2012]. The percentage of Frizzled 9+ cells was significantly increased in both conditions but with a higher portion in basal compared to standard conditions. This evidence suggests that BM may enrich stem-like cells, which are responsive to Wnt ligands. Moreover, it is possible to hypothesize that up-regulation of Frizzled 9 receptor may compensate the absence of growth factors in the maintenance of homeostasis. The low-affinity neurotrophins receptor p75, has been associated to neural progenitors [Kruger et al., 2002]. On the other hand, it is also expressed in glial lineage committed cells [Young et al., 2003; Sasselli et al., 2012]. Its expression was higher in SM than in BM cultures, suggesting that similar to SOX10 expression, the increase of p75 could be ascribed not only to a glial commitment but also to an expansion of neuronal progenitors. In order to better identify the neuronal subpopulation, PAN neuronal antigen expression was evaluated demonstrating to be present only in smaller cells (22.6 ± 3.1% in SM; 40.2 ± 3.5% in BM) (Figure 4.4). Although an increased number of total positive cells for TLR4 was detected in SM (40.8 ± 2.3%) respect to BM (26.8 ± 3.0%), a higher expression level of TLR4 was detected only in ENS

cultures unstimulated with growth factors (BM). This evidence suggest that the absence of growth factors, which simulate an agangliosis condition, up-regulates a cell population highly responsive to inflammatory stimuli. It has been reported that enteric neurons express TLR4 [Rumio et al., 2006] highlighting the presence of a TLR-based neural surveillance system in the intestinal wall [Barajon et al., 2009]. The *in vitro* simulation of a pathological condition, emphasizes this evidence suggesting that viral and bacterial agents could directly activate intestinal neural responses. No significative alterations of CD34 (31.3 ± 1.3% in SM; 33.7 ± 1.6% in BM) and Nestin (20.0 ± 1.8% in SM; 15.0 ± 1.7% in BM) expression were identified. Cells expressing CD34 are normally found in the umbilical cord and bone marrow as hematopoietic stem cells, a subset of mesenchymal stem cells, endothelial progenitor cells but also in a subset of differentiated endothelial cells [Holyoake et al., 1994; Asahara et al., 1997; Zvaifler et al., 2000]. As shown by Sanders and colleagues, the CD34 protein is expressed by stem cells of ICCs, considered as the pacemaker of the GIT, mediating the inputs of the ENS [Sanders et al., 1999]. The percentage of CD34+ cells was maintained over *in vitro* culture, independently from medium conditions, suggesting that this population may represent the stem-like cell pool. In fact, an important characteristic of stem cells is the maintenance of undifferentiated state. However, it is important to consider that CD34 can be associated either as a stem cell or ICCs and glial marker [Vanderwinden et al., 2000]. In order to better elucidate the presence of these CD34+ subpopulation further analysis considering co-expression with specific ICC and glial markers will be carried out. A slight increase of NG2 expression was observed in BM cultures (60.0 ± 3.8%) compared to SM (43.1 ± 0.8%). *In vitro* studies have suggested that NG2 participates in growth factor activation directly binding growth factors, such as bFGF and EGF [Goretzki et al., 1999]. These evidences suggest that NG2 may play an important role in organizing and presenting some types of mitogenic growth factors at the cell surface, and in condition of reduced availability of these factors an up-regulation of NG2 may be due to a

33

compensatory process. Although GFAP expression level unchanged in SM (15.3 ± 4.7%), a significative decrease (3.0 ± 0.4%) was detected in samples under basal conditions. If we consider GFAP as enteric glia marker, culturing under basal conditions showed to prevent differentiation.
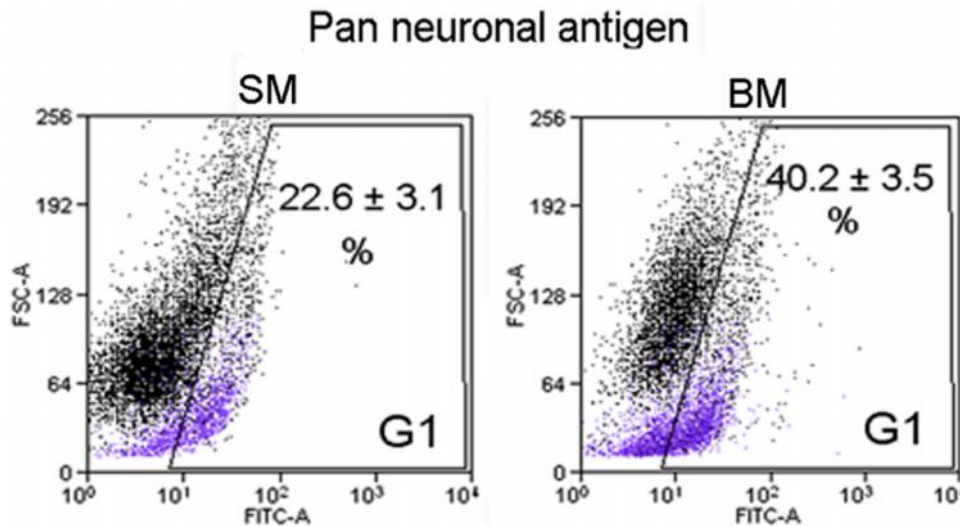

Figure 4.4 . Pan neuronal antigen expression of ENS cells cultured for 7 days in SM or BM. Data are reported as FSC vs Fluorescence dot plot using for R1 and R2 subsets blue and black colour, respectively. Positive expression of target markers is detected in gate G1 with respect to staining control; it is expressed as percentage (%) ± SD as measured manually or using the substraction/overlay statistics option of Summit 4.3 software (Beckman Coulter).

4.3 GFP Wnt reporter plasmid is a valuable tool for the detection of Wnt canonical pathway in ENS-derived cells

In order to define a useful system to detect by immunofluorescence canonical Wnt signalling-activated cells, a GFP Wnt reporter plasmid, named Top_EGFP3a, was experimented on ENS primary cultures. Since 24h from transfection, the expression of GFP was observed by optical fluorescence microscopy analysis in Top cells unstimulated and stimulated with rhWnt3a and LPS (Figure 4.5C-E) as well as in positive controls transfected with EGFP plasmid (Figure 4.5B). No signal was detected in controls prepared with empty vector (Figure 4.5A), demonstrating that auto-fluorescence did not occur. High transfection efficiency was observed by FCM at 96h (Figure 4.5G). In particular, GFP positive cells were about 80% and showed a decreased size (P1 population) in FCS/SSC dot plot (Figure 4.5F), as GFP is

34

reported to interact with cytoskeleton leading to cell size reduction [Agbulut et al., 2007]. A gradual accumulation of -catenin was observed in Top cells and negative controls from 24 to 72h (Figure 4.6A). The analysis by immunofluorescence showed a membrane (Figure 4.6B) and cytoplasmic (Figure 4.6C) localization of -catenin in ENS cells untransfected and transfected negative controls, respectively. This data suggest that a release of -catenin from plasma membrane to the cytoplasm was likely to be also induced in all samples by the transfection method. However, -catenin regulation by rhWnt3a (26.7 ± 0.72%) and LPS (39.6 ± 0.6%) stimuli was observed at 24h respect to negative control (55.9 ± 0.7%) and unstimulated Top cells (44.2 ± 0.4%) (Figure 4.6A), demonstrating an activation of Wnt signaling either by Wnt3a than LPS. In parallel, the analysis by confocal microscopy confirmed this activation, showing a nuclear localization of -catenin in Top cells (Figure 4.7) with coexpression of plasmid motif LEF1 in cytoplasm.
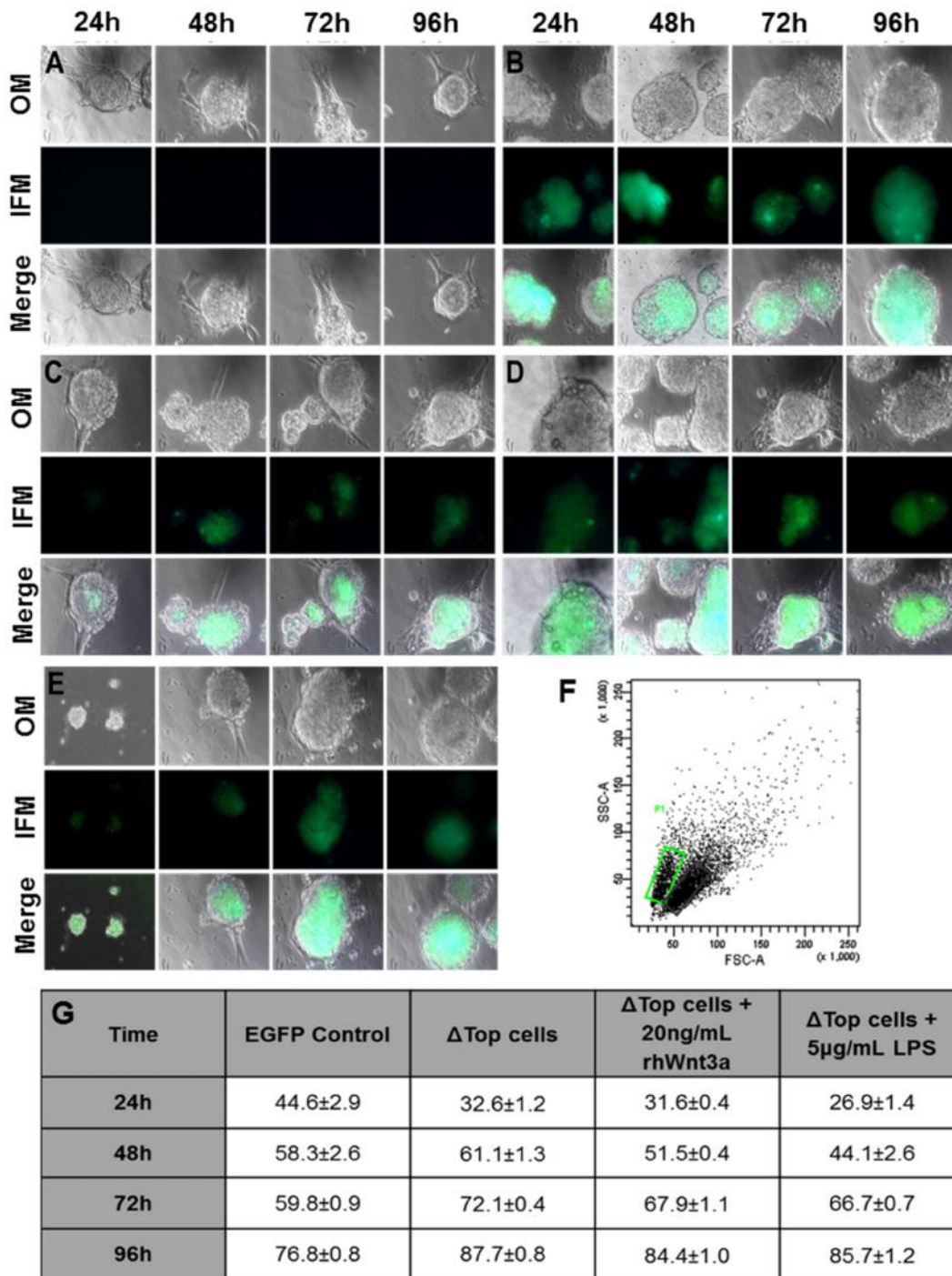
G Time	EGFP Control	ΔTop cells	ΔTop cells + 20ng/mL rhWnt3a	ΔTop cells + 5µg/mL LPS
24h	44.6±2.9	32.6±1.2	31.6±0.4	26.9±1.4
48h	58.3±2.6	61.1±1.3	51.5±0.4	44.1±2.6
72h	59.8±0.9	72.1±0.4	67.9±1.1	66.7±0.7
96h	76.8±0.8	87.7±0.8	84.4±1.0	85.7±1.2

Figure 4.5. Transfections were performed using an empty vector (A), either EGFP positive control (B) and a Wnt reporter plasmid (C-E). Top cells were stimulated with 20 ng/ml rhWnt3a (D) and 5 µg/ml LPS (E). FCM analysis of transfection efficiency was carried out in a time range between 24 and 96h. Data are expressed as percentage ± SD of positive cells with respect to controls prepared using isotopic or secondary antibodies (F). By FCM analysis two cell populations were displayed, characterized by different size and surface complexity (FCS vs SSC): the smaller population expressed GFP (population P1 green, F).
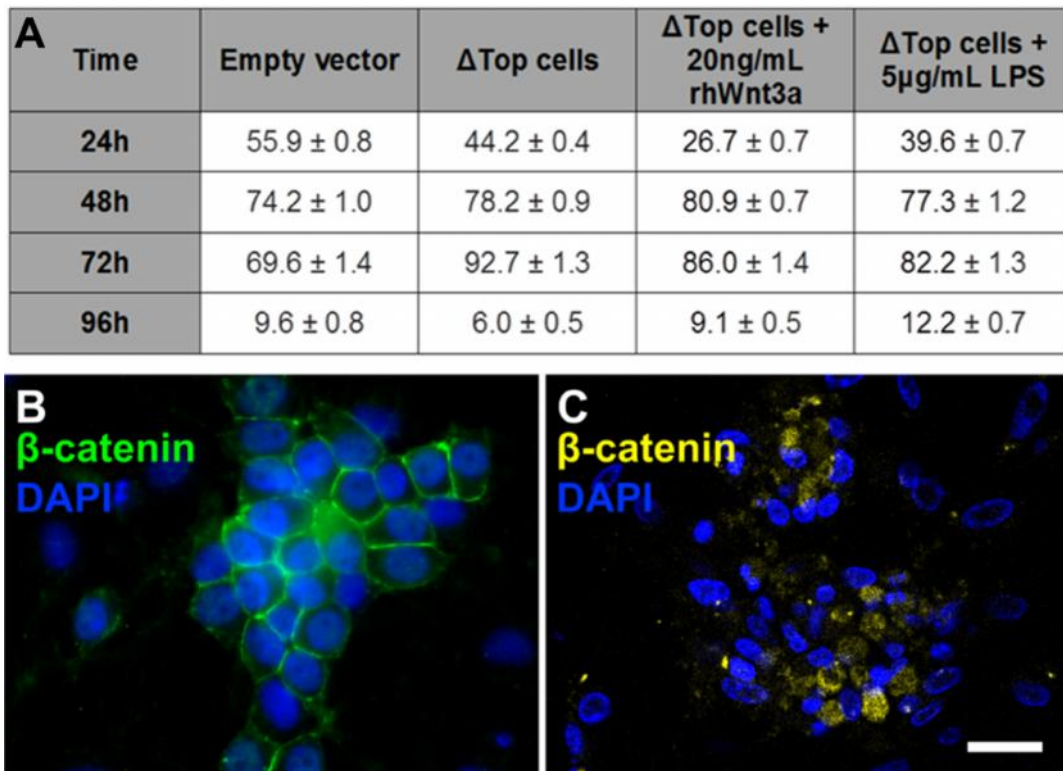
36

A Time	Empty vector	ΔTop cells	ΔTop cells + 20ng/mL rhWnt3a	ΔTop cells + 5µg/mL LPS
24h	55.9 ± 0.8	44.2 ± 0.4	26.7 ± 0.7	39.6 ± 0.7
48h	74.2 ± 1.0	78.2 ± 0.9	80.9 ± 0.7	77.3 ± 1.2
72h	69.6 ± 1.4	92.7 ± 1.3	86.0 ± 1.4	82.2 ± 1.3
96h	9.6 ± 0.8	6.0 ± 0.5	9.1 ± 0.5	12.2 ± 0.7

Figure 4.6. FCM analysis of -catenin accumulation in the time range 24-72h. Data are expressed as percentage ± SD of positive cells with respect to controls prepared using isotopic or secondary antibodies (A). Immunofluorescent staining of -catenin of untransfected (B) and transfected (C) cells indicated the release of -catenin into the cytoplasm due to the transfection method. Bar: 25 µm.
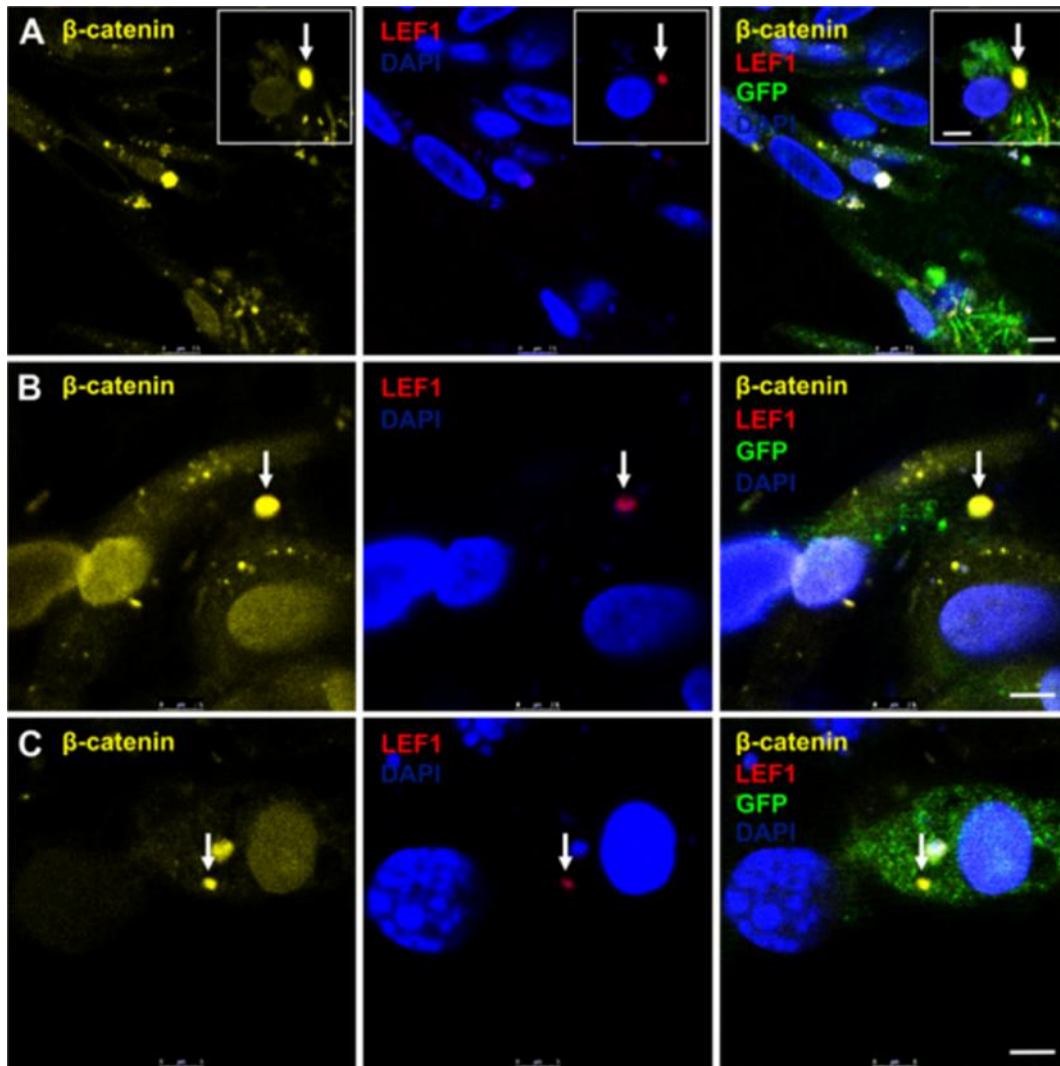
Figure 4.7. Confocal microscopy showing -catenin shuttling into the nucleus at 24h detected in transfected cells unstimulated (A), treated with rhWnt3a stimulated (B) and LPS stimulated (C). The staining was performed using -catenin (yellow) and LEF1(red) GFP expression (green) was observed in transfected cells. Bars: 5μm.

4.4 Wnt3a binds Frizzled-9 which is co-express with TLR4 on ENS subset

TLR4 and Frizzled-9 immunofluorescence staining performed on ENS cultured cells for 7 days in basal or standard conditions showed that a small size ENS subset co-express these two markers (Figure 4.8A, arrows). As previously demonstrated by FCM, small size cells can be referred to neuronal lineage cells (Figure 4.4). The immunofluorescence staining, showing an ENS subpopulation responsive to LPS and Wnt3a, suggests that a neuronal subset may represent a "key" cell in the maintenance of homeostasis and in the regulation of inflammatory processes. Moreover, co-immunoprecipitation demonstrated, for the first time, that Wnt3a specifically binds Frizzled-9 as shown by the 107 kDa western blot band (Figure 4.8B). Immunofluorescence staining shows also that big size ENS cells produce Wnt3a (Figure 4.8C). These data demonstrate that a controlled and specific cross-talk between the ENS subpopulations occurs.
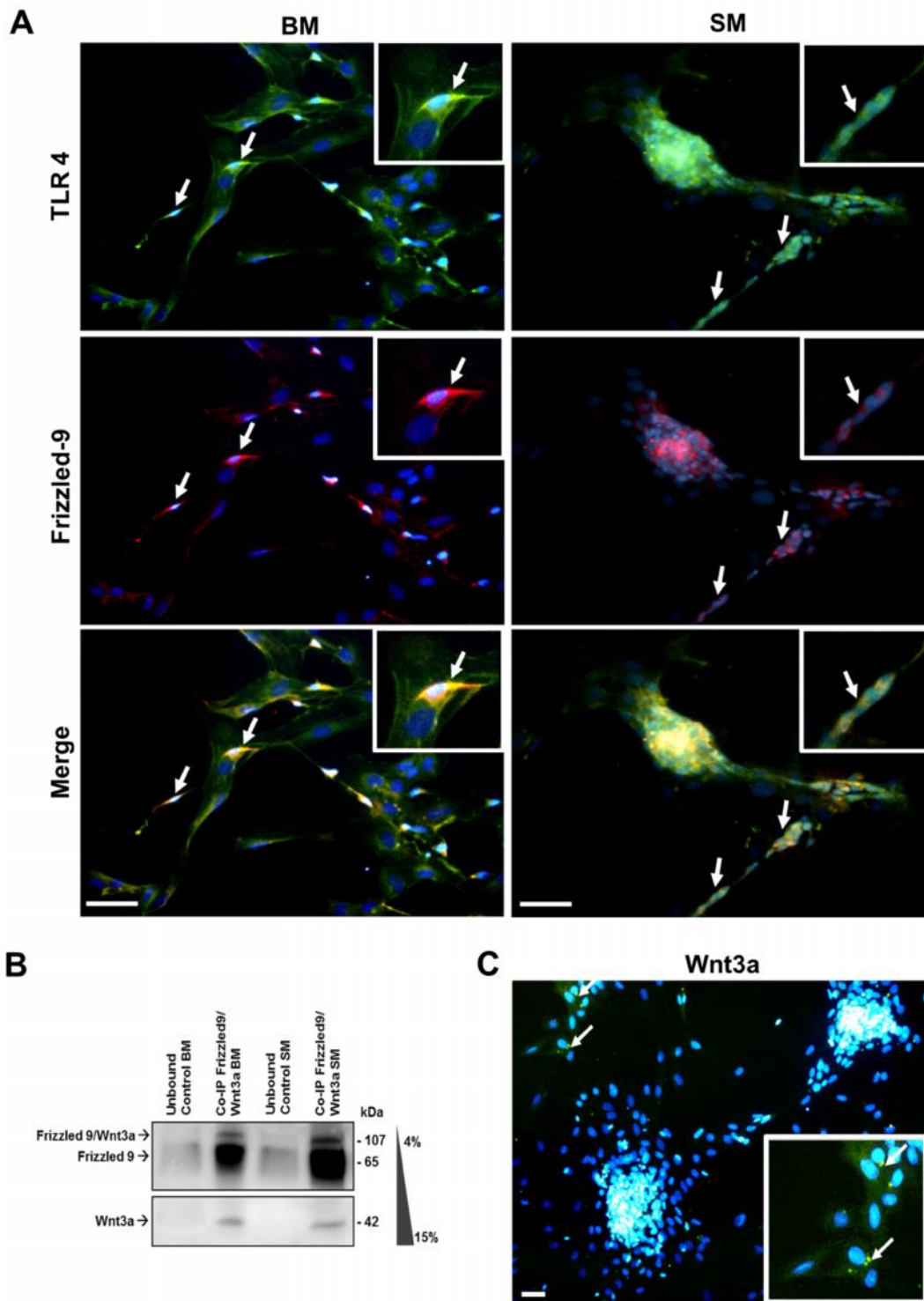
Figure 4.8. A) Confocal microscopy shows co-expression of TLR4 and Frizzled-9 (arrows) either in basal and standard medium cultured cells. B) Wnt3a and Frizzled-9 interaction is demonstrated by western blotting of co-immunoprecipitates performed on total protein extracts of ENS cells cultured for 7 days in BM or SM. Immunoaffinity purification was carried out using goat anti-rat Frizzled-9 and rabbit anti-rat wnt3a antibodies immobilized onto

Protein A-Sepharose. Western blot analysis was carried out with a 4-15% gradient precast gel. C) Immunofluorecence performed on ENS cells stained with rabbit ant-rat Wnt3a antibody. Arrows are showing vescicles containing Wnt3a. Bar: 15 µm.

4.5 Canonical Wnt pathway is active in ENS cells and is continuously stimulated under standard culture conditions

The canonical pathway of Wnt signalling and LPS/TLR4 pathway play important role in controlling the enteric plasticity. To evaluated the cross-talk between these two pathways, a western blot analysis of pAkt, pGSK-3 and phosphorilation/traslocation of -catenin was carried out using the unstimulated ENS cells as reference and a time course ranging from 0 to 24h. Under SM conditions, the detection of p-GSK-3, p-Akt and nuclear -catenin at T0 and their variable expression within 24h demonstrated an important modulation by growth factors on Wnt and LPS pathways (Figure 4.9 and Figure 4.10). In contrast, no activation of Wnt and LPS signalling was detected in BM-treated samples at T0 (Figure 4.9) suggesting that BM condition was more useful to discriminate the specific effects of Wnt3a and LPS on ENS cells. The presence of p-Akt and the traslocation of -catenin and Nf- B were observed in unstimulated BM-samples from 30' to 24h (Figure 3.9A and Figure 4.11). Under stimulation with rhWnt3a, a regulation of p- -catenin, p-Akt and p-GSK-3 at early phase (1-2h) was observed (Figure 4.9B). Moreover, a cyclic regulation of p- -catenin through GSK-3 was detected at intervals of 4h (Figure 4.11) and the delayed traslocation of Nf- B at 2h suggested a negative regulation by Wnt stimulus (Figure 4.9B). As the nuclear traslocation of Nf- B was detected at 30' and a similar expression panel of Wnt signaling components was observed after treatment, LPS was hypothesized to interact early with Wnt pathway probably involving p-Akt (Figure 4.9C), as previously demonstrated by Fang et al [2007].
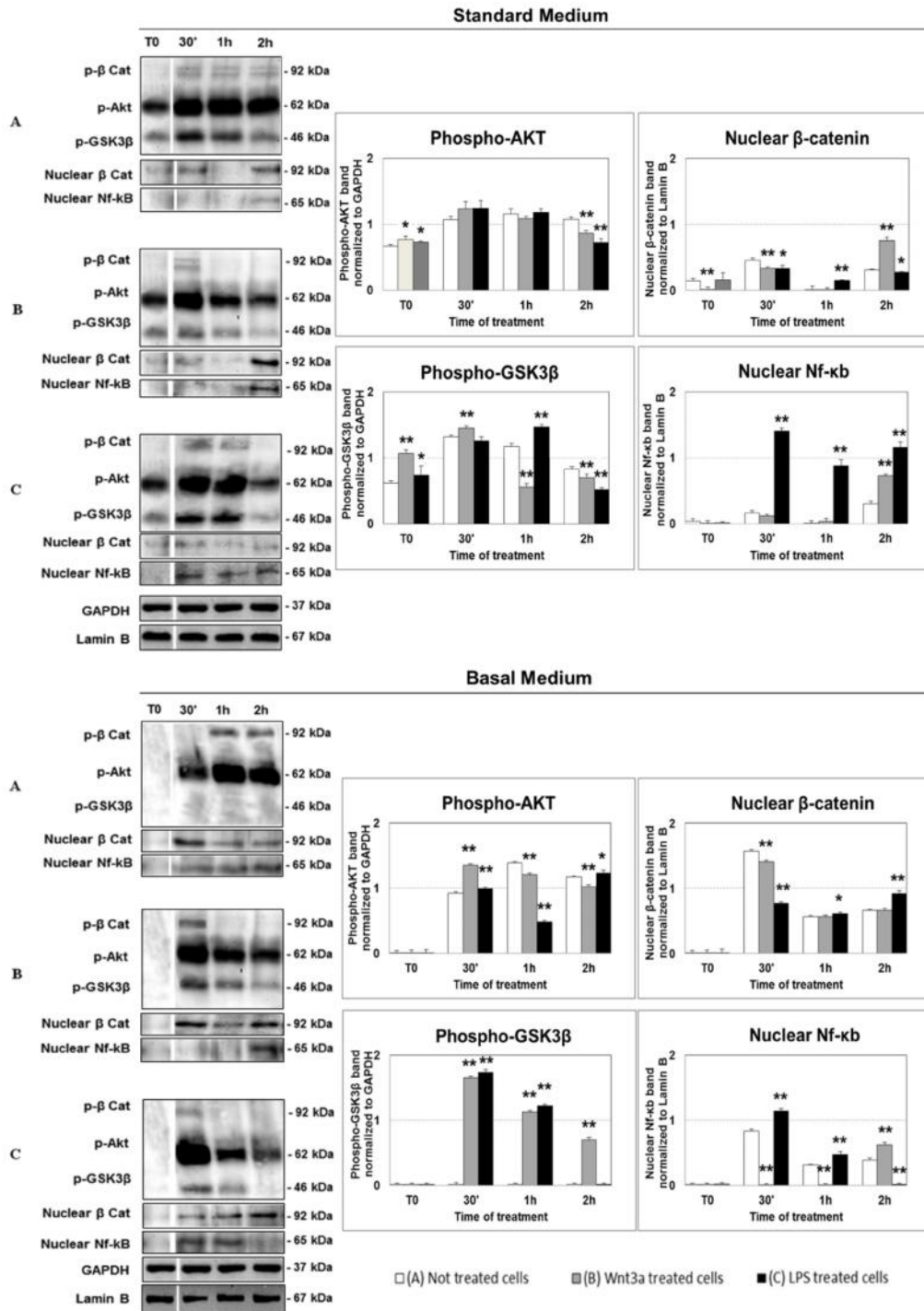
Figure 4.9. Western blot analysis at T0, 0.5-2h using antibodies specific for cytoplasmatic phosphorylated form of Akt, GSK-3, -Catenin and nuclear localization of -Catenin and Nf-κB in unstimulated cells (A), cells treated with rhWnt3a (20 ng/mL) (B) and LPS (5 µg/mL) (C) in SM and BM. GAPDH and lamin B were considered as cytoplasmatic and nuclear housekeeping protein, respectively. Quantification of protein expression was performed using the image processing software ImageJ. Data are reported as ratio within target protein and relative housekeeping protein expression. Statistical significance, calculated by t-Test, compared to control: p-value 0.05: *, p-value 0.01: **.
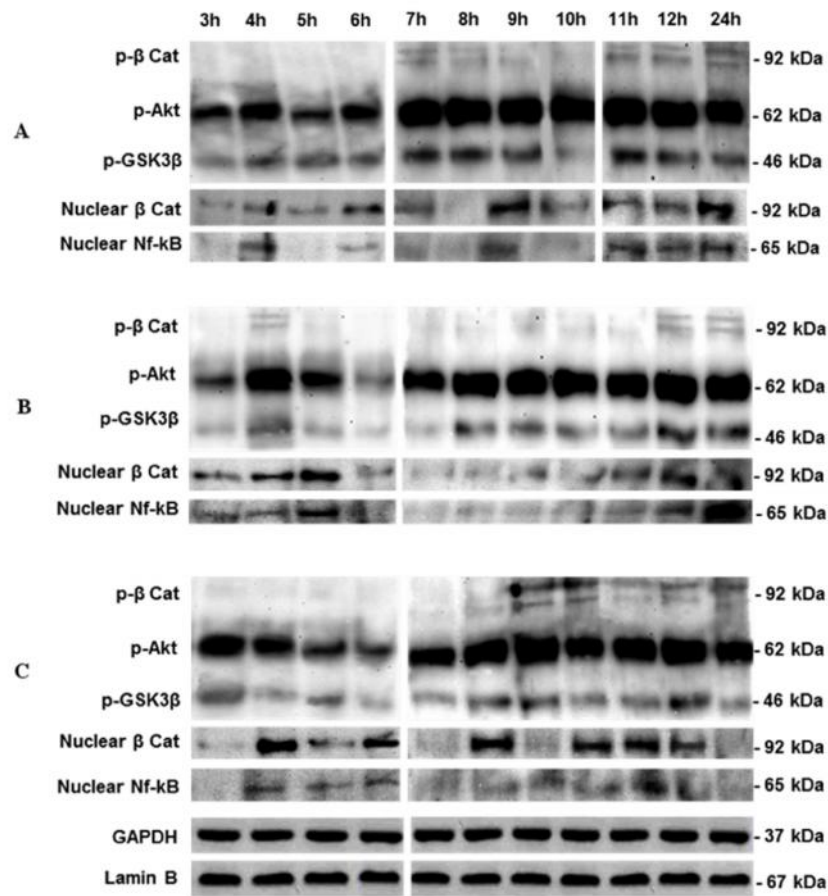
Figure 4.10. Western blot analysis from 3h to 24h using antibodies specific for cytoplasmatic phosphorylated form of Akt, GSK-3 , -Catenin and nuclear localization of -Catenin and Nf-B in unstimulated (A), rhWnt3a (20 ng/mL) (B) and LPS (5 µg/mL) (C) stimulated ENS cells in SM. GAPDH and lamin B were considered as cytoplasmatic and nuclear housekeeping protein, respectively.
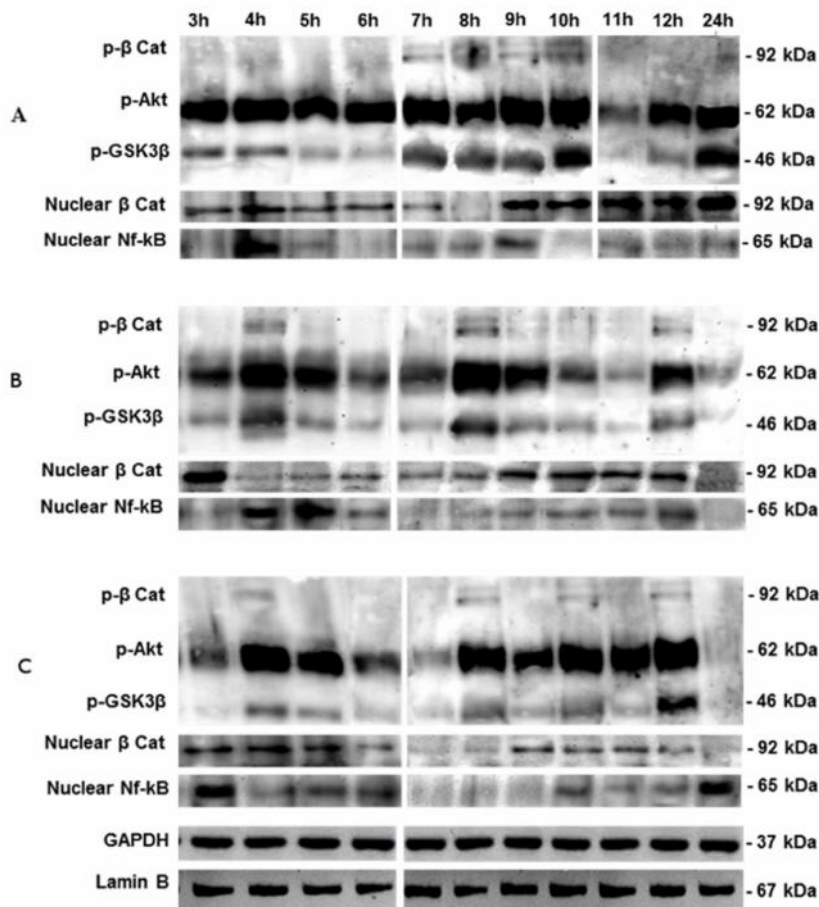
43

Figure 4.11. Western blot analysis from 3h to 24h using antibodies specific for cytoplasmatic phosphorylated form of Akt, GSK-3 , -Catenin and nuclear localization of -Catenin and Nf-B in unstimulated (A), rhWnt3a (20 ng/mL) (B) and LPS (5 µg/mL) (C) stimulated ENS cells in basal medium. GAPDH and lamin B were considered as cytoplasmatic and nuclear housekeeping protein, respectively.

4.6 Exogenous Wnt3a exerts anti-inflammatory activity on ENSc

The effects of Wnt3a and LPS on gene expression of ENS cells cultured under BM were evaluated using One Step® RT-PCR and qPCR. As suggested by the expression of specific mRNAs (Figure 4.12), a basal expression of GDNF, EGF, FGF2, NGF and LIF was observed in all samples without any strong modulation by culture and stimulation conditions. As reported previously, these factors may interfere with LPS and Wnt signaling. For this reason, it is important to consider that after 7 days, the effect of Wnt3a and LPS might be not specifically defined as a cross-talk of both

signalling systems. Interference to the pathways by released endogenous factors could not be excluded. Thus, the real effect of Wnt3a and LPS can be observed at 24h from stimulation.
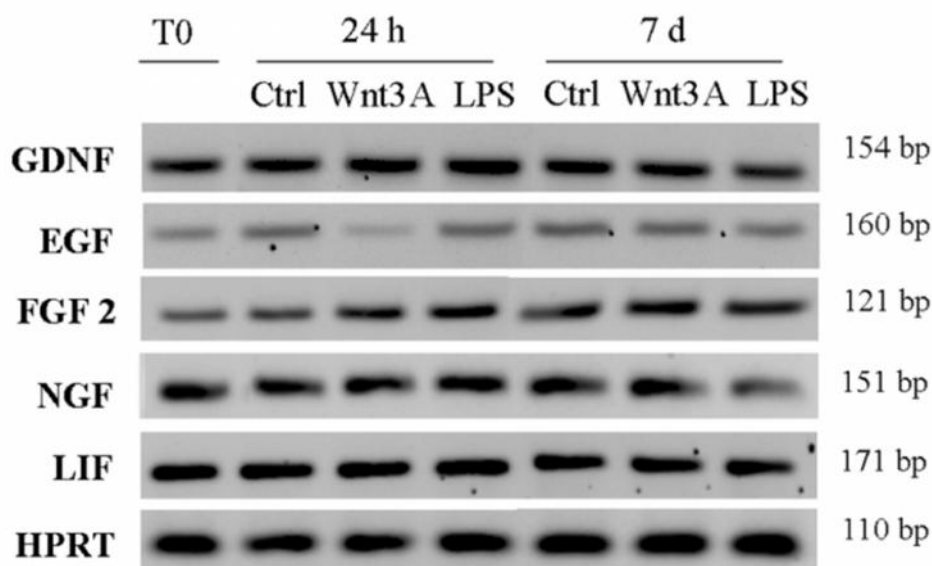

Figure 4.12. RT-PCR, performed using Qiagen One Step RT–PCR Kit, of rhWnt3a (20 ng/mL) and LPS (5 µg/mL) stimulated cells after 24h and 7 days compared to unstimulated cells (Crtl) and T0. Total RNA considered for the analysis was 10 ng and RT–PCR products were electrophoresed on a 2% agarose gel. GDNF, EGF, FGF2, NGF and LIF were considered as target gene compared to housekeeping HPRT expression.

As reported in Figure 4.13, the role of Wnt3a and LPS in ENS Wnt pathway was elucidated by qPCR. As AXIN-2, c-jun, c-myc are reported as target genes of -catenin transcriptional activity, the upregulation (p 0.01) of their expression at 24h was considered dependent on the activation of Wnt signaling in comparison with control. Interestingly, LPS treatment increased the expression of c-jun (p 0.01) and AXIN-2 (p 0.05), which are under the control of -catenin, demonstrating a cross-talk between LPS and Wnt pathways. After 7 days these -catenin transcriptional genes were upregulated in LPS-treated cells (c-myc and c-jun p 0.05; AXIN-2 p 0.01). C-myc has been reported to be also regulated by Nf- B [Duyao et al., 1990]. When ENS cultures were treated with LPS, at 24h downregulation of TLR4 (p 0.05) was observed whereas rhWnt3a up regulated this receptor either alone (p 0.05) than in combination with LPS (p 0.01). FZD9 mRNA

expression was increased with both stimuli (p 0.05). LPS treatment, alone or together with rhWnt3a, increased the expression of Wnt3a (p 0.01) (Figure 4.13A), suggesting a role of this ligand in the regulation of immune response. Liu and colleagues [2012] demonstrated that Wnt2 contributes to host protection in response to enteric bacteria in the intestine epithelial cells. Compared to control, at 24h an up-regulated LPS-induced expression was observed for CCL3 (p 0.01), IL-1 (p 0.01), IL-6 (p 0.01) and TNFα (p 0.01) demonstrating an acute inflammatory effect mediated by Nf- B. In contrast, a downregulation of these markers occurred in Wnt3a-stimulated cells (IL-6 p 0.01; IL-1 p 0.01; CCL3 p 0.01). Moreover, anti-inflammatory IL-10 was up-regulated by rhWnt3a stimulation (p 0.01), whereas a significative (p 0.05) downregulation was observed in LPS-treated cells. Anti-inflammatory effect of Wnt3a was observed also in double treated cells as demonstrated by the down regulation of IL-1 (p 0.01), the significant reduction of IL-6 respect to LPS-treated cells (p 0.01) and the up-regulation of IL-10 (p 0.01). This anti-inflammatory effect was maintained after 7 days as demonstrated by IL-1 (rhWnt3a p 0.01 and double treated p 0.01) and by IL-10 up regulation in double treated cells (p 0.01). No effect of Wnt3a was observed on CCL3 expression in double treated cells, suggesting that LPS stimulation may be more effective for the mRNA regulation of this marker. After 7 days, no expression of IL-6 and CCL3 was observed, probably due to a differential expression time. RT–PCR products of CCL3 separated by electrophoresis showed a mRNA alternative splicing regulation represented by about 400 bp bands [AceView, 2006] (Figure 4.13B). This alternative splicing regulation generates a non-coding mRNA suggesting that Wnt3a may contrast the inflammatory chemokine CCL3 at transcriptional level.
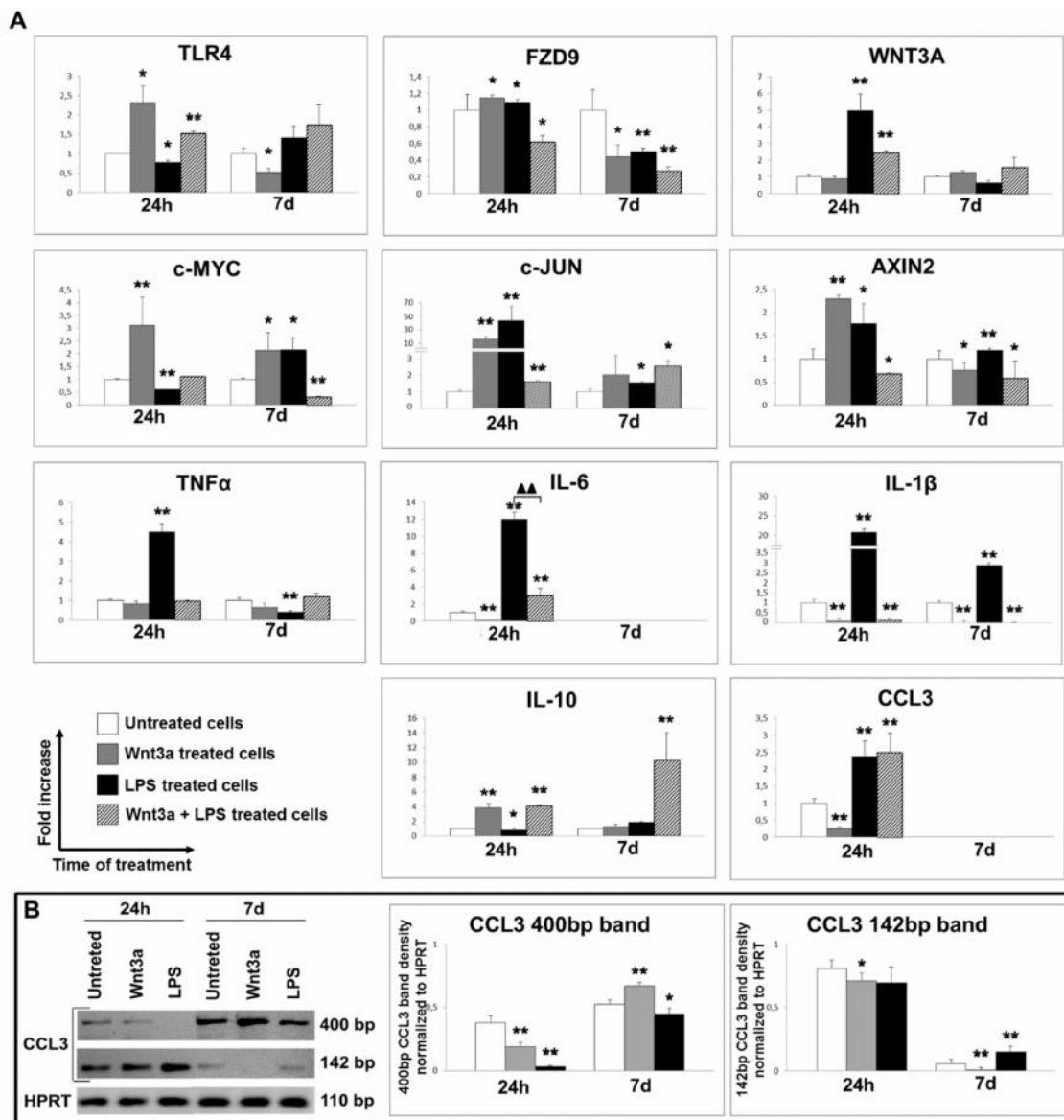
Figure 4.13. Gene expression study by Real-Time PCR in ENS cells treated with rhWnt3a (20 ng/mL) and LPS (5 µg/mL). Amounts of each gene product were calculated using linear regression analysis from standard curves, demonstrating amplification efficiencies ranging from 90 to 100%. The term "fold increase" is defined as the cDNA ratio between target gene and reference gene (HPRT) normalized to untreated control. A) mRNA expression of β-catenin target gene (AXIN-2, CMYC and C-JUN), receptors (TLR4 and FZD9), WNT3A ligand and pro and anti-inflammatory target genes (CCL3, IL-1β, IL-6, TNFα and IL-10). B) RT–PCR products of CCL3 were electrophoresed on a 2% agarose gel showing an alternative splicing regulation (400bp bands). Quantification of mRNA expression was performed using the image processing software ImageJ. Data are reported as ratio within target band density and relative housekeeping band. Statistical significance, calculated by t-Test, compared to untreated: p-value ≤ 0.05: *, p-value ≤ 0.01: **. Significance compared to LPS treated cells: p-value ≤ 0.01: ▲.

4.7 Small bowel completely decellularizes after 5 cycles of detergent-enzymatic treatment.

In order to completely remove the cells present in the small intestine of adult rat, 5 cycles of decellularization were performed. The morphological changes were observed by toluidine blue staining, while the successful decellularization was verified by quantification of nucleic acids (RNA and DNA) and staining of nuclei with DAPI. The decellularization process reduced principally the thickness of luminal layer, resulting in the loss of the characteristic intestinal mucosa villi. After 4 cycles of decellularization still few nuclei were observed in the luminal side, while a further cycle determined a complete tissue decellularization (Figure 4.14A). The quantification of nucleic acids confirmed the progressive cells loss as the number of cycles increased (Figure 4.14B).
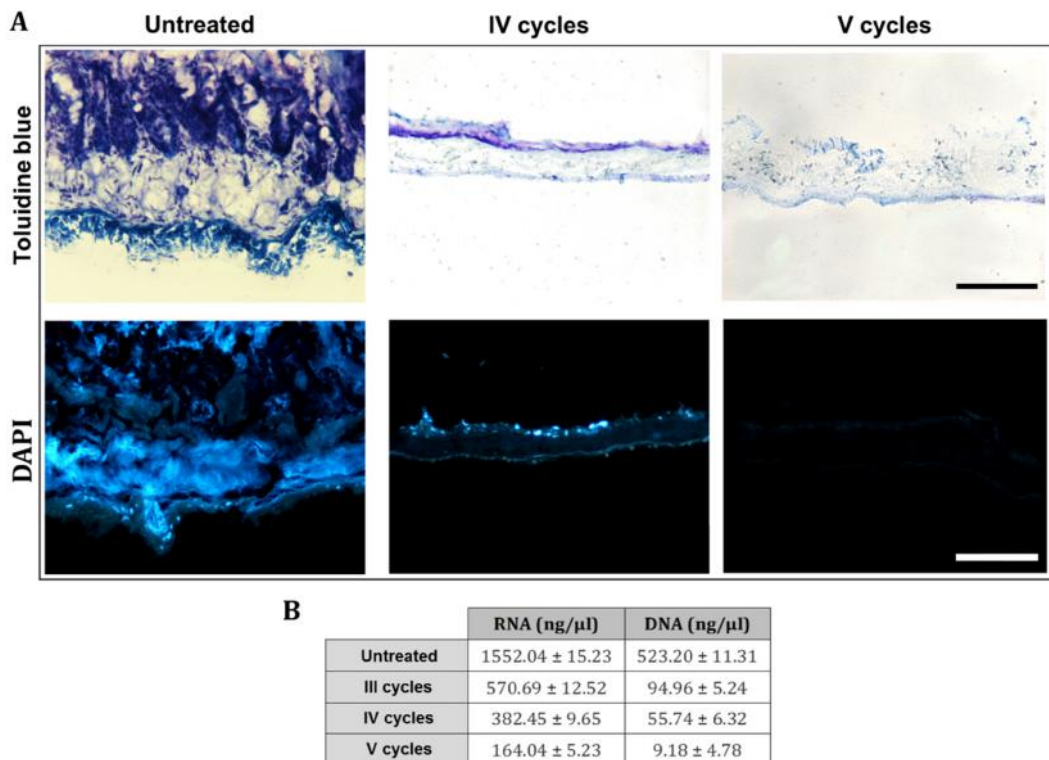

	RNA (ng/µl)	DNA (ng/µl)
Untreated	1552.04 ± 15.23	523.20 ± 11.31
III cycles	570.69 ± 12.52	94.96 ± 5.24
IV cycles	382.45 ± 9.65	55.74 ± 6.32
V cycles	164.04 ± 5.23	9.18 ± 4.78

Figure 4.14. A) Morphological analysis by toluidine blue and DAPI staining of untreated bowel and AM decellularized by four and five cycles. Bar: 25 pm. B) RNA and DNA quantification (ng / µl ± SD) of AMs (III, IV, V cycles) compared to untreated tissue.

The morphological analysis by scanning electron microscope (SEM) was performed both on the outer than the luminal side (Figure 4.15). Not treated outer layer showed a flat and homogeneous surface, while the luminal side was characterized by the presence of villi and crypts. Following treatment with dispase II, cell loss in both sides was observed. In particular, the crypts were partially covered by cellular debris and the fibrillar structures of the extracellular matrix began to be evident in the outer side. After five cycles, these structures were more obvious and the crypts appeared deeply clear.
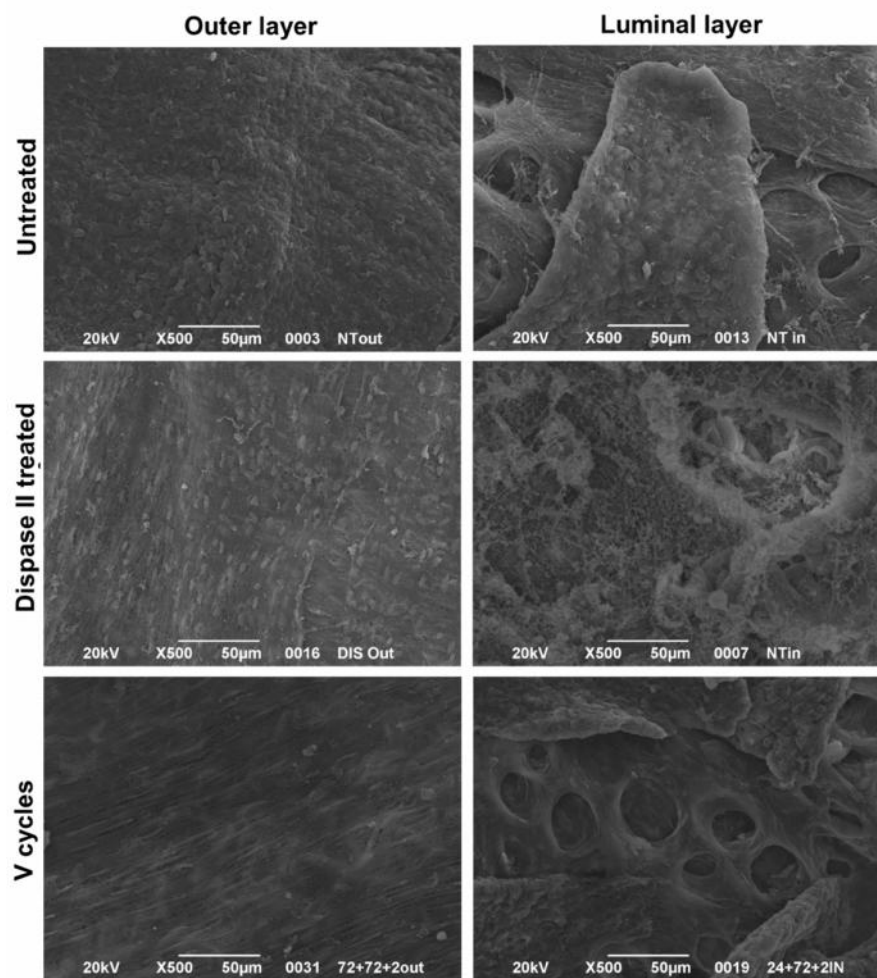

Figure 3.15. SEM of native and decellularized bowel tissue.

The protein content of the acellular matrix obtained after 5 cycles of decellularization was assessed by immunofluorescence and western blot (Figure 4.16). The type I collagen appeared diffuse throughout the AM as

precursor (130 kDa) and mature form (90 kDa), whereas Collagen III was distributed mainly in the luminal side. The AM contained components of the basal membrane, such as laminin, localized in both sides but not into the inner portion, and type IV collagen, present as a 90 kDa isoform.
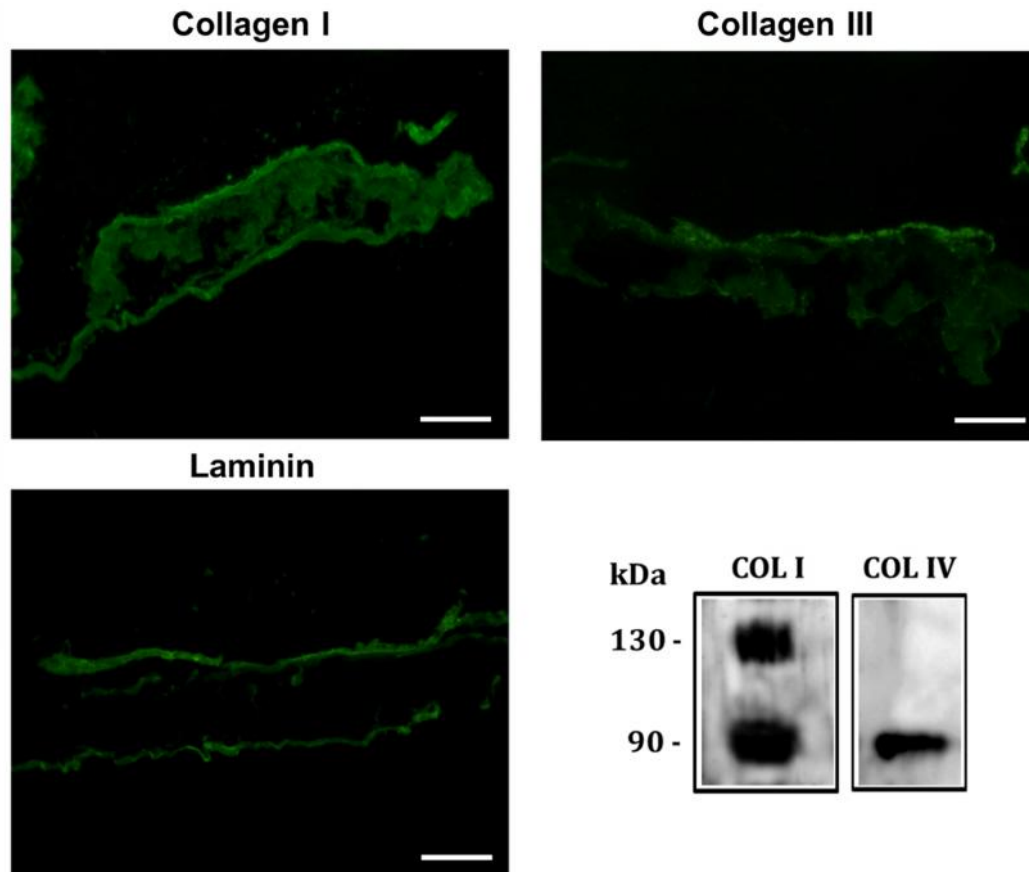

Figure 4.16. Protein characterization of AM (5 cycles) by immunofluorescence and western blot. Bar: 25 pm.

4.8 The acellular matrix modulates the organization of ENS-derived cells

The cells cultured for 7 days in SM or BM were seeded on AMs. After 3, 7 and 14 days, the morphology and the expression of specific differentiation markers were evaluated by SEM, immunofluorescence and RT-PCR, respectively. The AM cultures, maintained in BM, had flattened and partially stratified cellular elements (Figure 4.17). Neuronal differentiation did not occur, as demonstrated by the absence of III tubulin. However, -SMA, a glial and smooth muscle cell marker, was expressed and increased with time in culture.
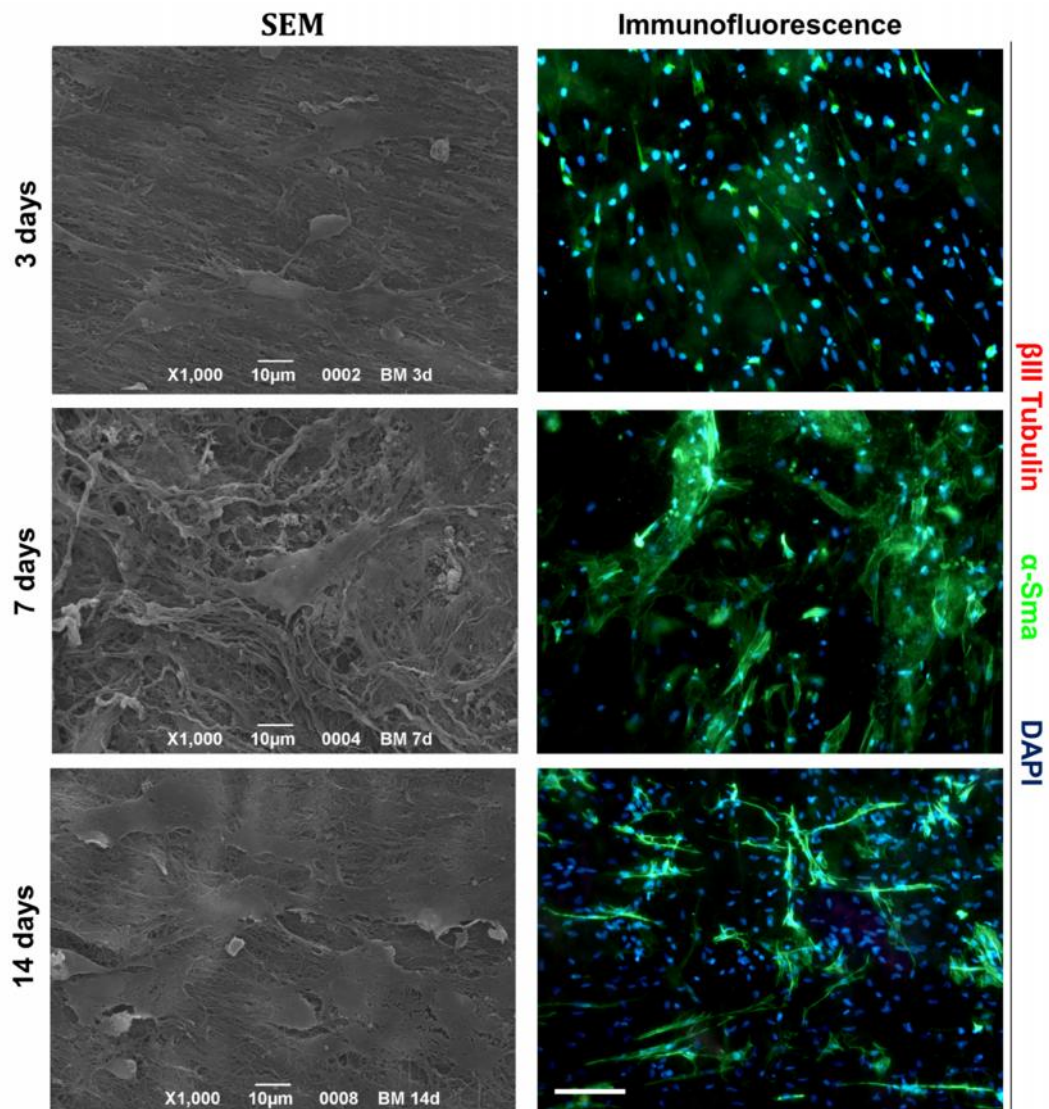
Figure 4.17. SEM and immunofluorescence analysis of neuronal markers (βIII tubulin, red) and glial / muscle (α-SMA, green) performed on AM cultures with ENS-derived cells cultured in BM. Bar: 25 μm.

In contrast, after 7 days with standard medium, AM cultures were composed of different cells: flattened elements that formed a monolayer and, on the latter, small clusters of cells characterized by small size and round shape. In particular, after 14 days, these aggregates evolved in ganglion-like structures, suggesting a neuronal differentiation. Furthermore, these structures were connected by tubular formations simulating enteric neural network (Figure 4.18).

SEM standard medium
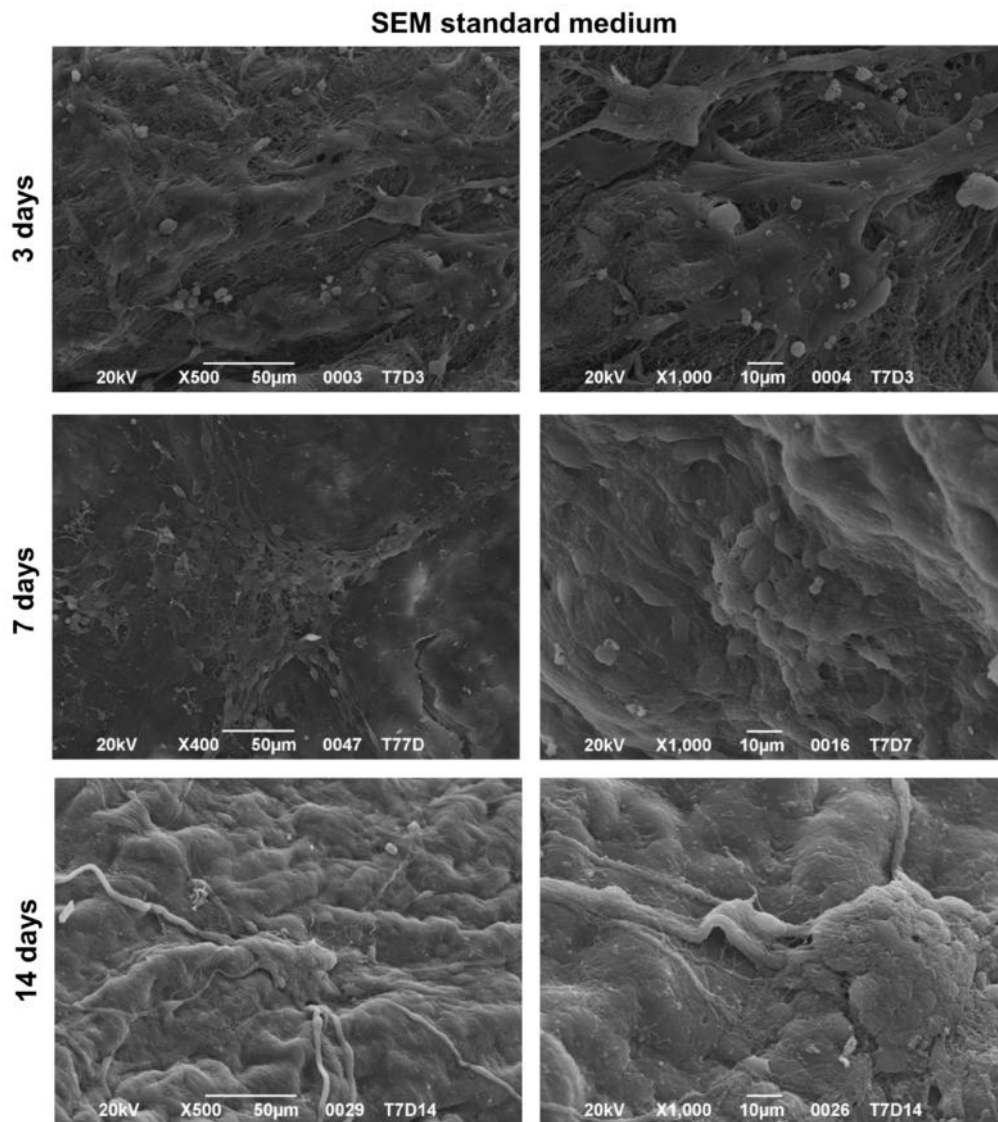
Figure 4.18. SEM analysis of AM cultures with ENS-derived cells cultured in SM.

The neuronal differentiation was confirmed by the presence of III tubulin, whose expression increased with time in culture coinciding with the ganglion-like structures and their connections. In contrast, cells that formed the monolayer were -SMA-positive and did not express III tubulin (Figure 4.19A). Similarities were observed comparing the expression of III tubulin and -SMA in native myenteric plexus and AM cultures (Figure 4.19B).

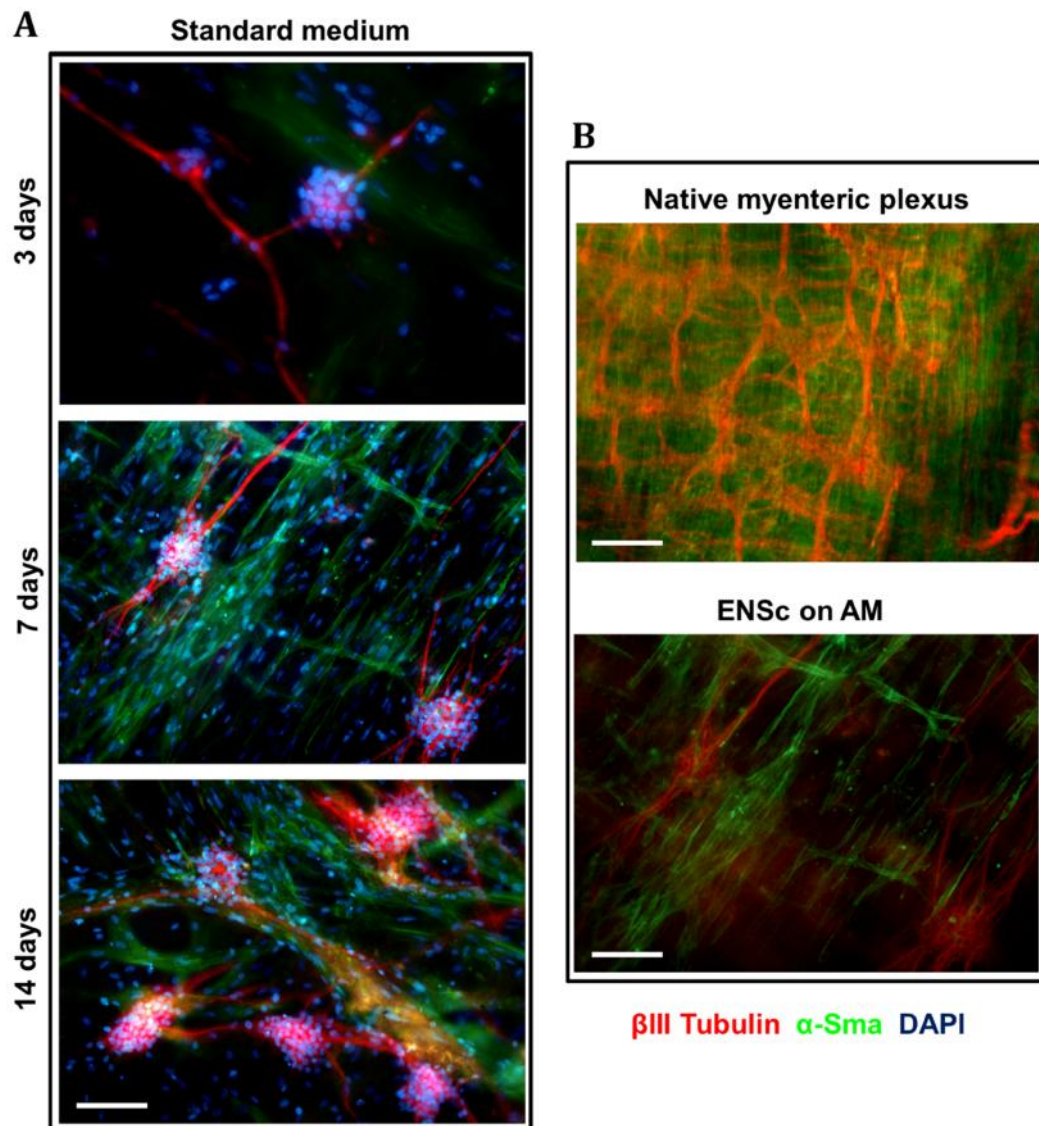**Figura 4.19.** A) Immunofluorescence analysis of neuronal markers (βIII tubulin, red) and glial/muscle (α-SMA, green) of AM (V cycles) repopulated with ENS cells cultured in SM. B) Comparison of βIII tubulin (red) and α-SMA (green) expression in native myenteric plexus and in AM cultures after 14 days in culture in SM. Bar: 25 μm.

The expression of glial (S100B), neuronal (βIII tubulin) and undifferentiated state (SOX2) markers was evaluated by RT-PCR. No modulation of these markers was observed in AM cultured with SM, whereas basal conditions showed a decreased expression of these markers, mainly at 7 and 14 (Figure 4.20). These results showed that the matrix is able to induce a more complex cellular organization compared to that observed in cultures on polystyrene. In fact, even in the presence of complete medium which, as mentioned above

provides a greater amount of differentiation stimuli, the cells seeded on polystyrene formed isolated neurospheres. In contrast, the AM cultures were organized in ganglion-like structures partially interconnected and lying on a substrate of cells which may function as support. In fact, as demonstrated by immunofluorescence, these cells were positive for -SMA, a glial and smooth muscle marker. Several studies, performed on cell cultures, have emphasized the crucial role of the various components of the ECM, including laminin, both in cell migration and in the development of neurites. Moreover, microenvironment signals are necessary for the formation of enteric ganglia promoting NCCs differentiation into neurons [Sanes, 1989; Neugebauer et al., 1991; Lallier et al., 1991].
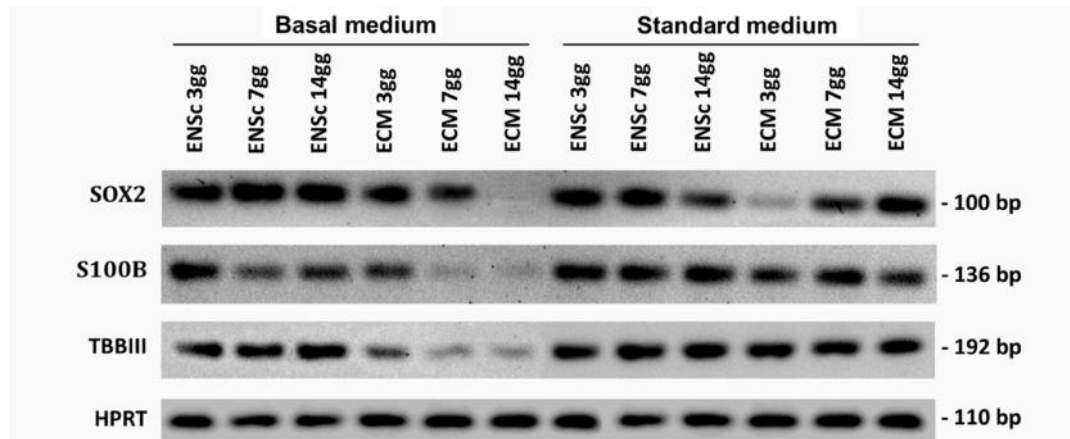

Figura 4.20. RT-PCR analysis performed on ENS-derived cells cultured in BM or SM on AM or polystyrene. Total RNA considered for the analysis was 10 ng and RT–PCR products were electrophoresed on a 2% agarose gel. SOX2, S100B and III tubulin (TBBIII) were considered as target gene compared to housekeeping HPRT expression.

5. Conclusion

Microenvironmental stimuli plays an important role in the development, homeostasis maintenance and differentiation. The influences of soluble factors, Wnt signaling as well as ECM interaction have been well characterized and studied in the ENS development. Little is known about the role of these microenvironmental factors in the adult ENS. Taken together, our data showed that ENS-derived cells respond to different culturing conditions modulating their immunophenotype. Moreover, Wnt signaling was active in post-natal ENS and a cross-talk with LPS/TLR4 pathway was demonstrated. Furthermore, a neuronal population, co-expressing the receptors Frizzled-9 and TLR4, was characterized and may represent a "key" cell population involved in the maintenance of homeostasis and in the regulation of inflammatory processes. The stimulus with Wnt3a demonstrated to be anti-inflammatory, and the autologous production by ENS glial-like cells of this ligand suggested a fine interaction between ENS-derived cells aimed to control inflammation. The extracellular matrix affected the spatial organization of the ENS cells and exerted a synergic effect with the factors present in the culture medium. Finally, ganglion-like structures partially interconnected suggest that ENS cells cultured on AM may represent a useful *in vitro* model for toxicological and pharmacological studies as well as a possible biomaterial in regenerative medicine.

6. List of Abbreviations

The following abbreviations have been used in this thesis

APC	Adenomatous polyposis coli
AM	Acellular matrix
B-FABP	Brain Fatty Acid Binding Protein
BM	Basal medium
-TrCP	-transducin repeat-containing protein
CCL3	Chemokine (C-C motif) ligand 3
ChAT	Choline acetyltransferase
CFU	Colony forming unit
CK1	casein kinase 1
CNS	Central nervous system
CPM	Complete proliferation medium
DAPI	4',6-diamidino-2-phenylindole
DSH	Dishevelled
ECM	Extracellular matrix
EGF	Epidermal growth factor
ENS	Enteric nervous system
ENCC	Enteric neural crest cell
ESC	Embryonic stem cell
FITC	Fluorescein isothiocyanate
FGF	Fibroblast growth factor
GAG	glycosaminoglycans
GDNF	Glial cell-line derived neurotrophic factor
GIT	Gastro-intestinal tract
GFAP	Glial fibrillary acidic protein
GSK3	Glycogen synthase kinase 3
IBD	Inflammatory bowel disease
IL	Interleukin

ICC	Interstitial cells of Cajal
LEF	Lymphocyte enhancer factor
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharides
Mash1	Mammalian achaete-scute homologue-1
MAPK	mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
NC	Neural crest
NF-κB	nuclear factor-κB
P3	Postnatal day 3
PBS	*Phosphate Buffered Saline*
PI3K	phosphatidylinositol 3 kinase
PCR	Polymerase chain reaction
PGP 9.5	Protein gene product 9.5
PNS	Peripheral nervous system
RET	Receptor tyrosine kinase gene
SMA	Smooth muscle actin
SOX	Transcription factor containing an SRY-related HMG-box
SVZ	Subventricular zone
TCF	T-cell factor
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor-α
LBP	Lipopolysaccharide binding protein
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
MyD88	Myeloid differentiation primary response 88
IRAK	Interleukin-1 receptor-associated kinase 1
RIP2	Receptor-interacting serine/threonine-protein kinase 2
AKT or PKB	Protein Kinase B
NGF	Neural growth factor
NCCs	Neural crest cells

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