Chapter 11

Biodegradable Scaffolds for Gastric Tissue Regeneration

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Abstract

Tissue engineering has been viewed as a valid approach toward the partial or total replacement of defective tissues and organs. Recent advances in nanotechnology have made it possible to develop biocompatible materials at the micro- and nano-scales to be used as scaffolds for cellular growth and regeneration of defective tissues. Gastric mucosal lining is an example of soft tissues that are highly susceptible to damage due to various reasons including cancer or ulcer development. Current therapeutic approaches to these diseases have some limitations. This chapter describes the basis for development of a novel modality combining nanotechnology, stem cells, and tissue engineering for the replacement of defective gastric tissues using synthetic biocompatible scaffolds. These microfibrous scaffolds are seeded with gastric stem cells, which are studied for their proliferation and differentiation into functional gastric mucous cells.

Keywords: gastric stem cells, mucous neck cells, electrospinning, porous microfibrous scaffold, tissue regeneration

1. Introduction

Gastric cancer remains the second or third largest cause of cancer-related mortality worldwide [1, 2]. The standard operation for early or advanced gastric cancer is partial or radical gastrectomy. Patients after gastrectomy suffer from various complications such as dumping syndrome and pernicious anemia. These conditions are attributed to the loss of storage, digestive, and exocrine glandular functions of the stomach such as secretion of gastric enzymes, acid and intrinsic factor [3]. More than two-third of gastric cancer cases are unresectable and their response rate to chemotherapy is very low. Cases subjected to gastrectomy have less than 30% chance of 5-year survival [4, 5].

The stomach is the most dilated part of the digestive tube, which connects the esophagus with the small intestine (**Figure 1**). The wall of the stomach comprises four coats: serosa, musculosa, submucosa, and mucosa. The outermost serosa layer represents the peritoneal covering of the stomach. The luminal surface of the stomach has innumerable micro-openings for gastric pits (foveolae) continuous with the isthmus, neck and base of the gastric glands. In the cardia and pyloric regions, these glands are mostly populated by mucous cells and some enteroendocrine cells. However, the corpus glands are populated by two different types of mucous cells (pit and neck cells), pepsinogen-secreting chief or zymogenic cells, acid-secreting parietal cells, and hormone-secreting enteroendocrine cells (**Figure 1**). All these cells originate from stem cells residing in the isthmus region of the gastric glands. In addition to the role of these stem cells in physiological maintenance of the heterogeneous epithelium of the gastric gland, these cells are necessary for regeneration, healing, and repair of the gastric mucosa.

Although some gastric replacement approaches have been proposed to improve the quality of life of patients after gastrectomy, the optimal reconstruction procedure remains controversial [6]. Recent advances in the field of tissue engineering allowed fabrication of many tissues and organs. As an alternative remedy to the post-gastrectomy complications, gastric mucosal tissue engineering has been proposed. It has long been believed that the stomach never regenerates once it has been resected [7]. However, tissue-engineered stomach is an attractive solution post-gastrectomy to restore an adequate food digestion and appropriate gastric physiology.

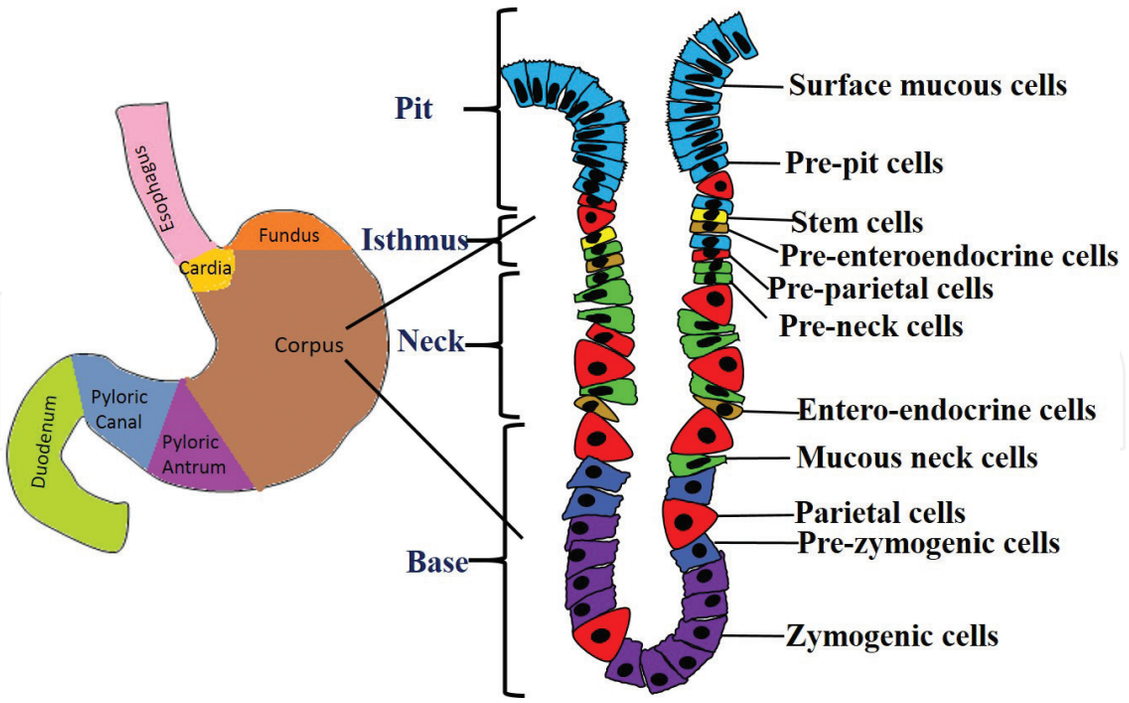

Figure 1. Diagrams depicting the structure of the stomach and gastric epithelial unit including the pit and three glandular regions: isthmus, neck and base. Note that stem cells and their immediate descendants are located in the isthmus. Surface mucous, mucous neck, and zymogenic cells are located in the pit, neck, and base, respectively. Parietal and enteroendocrine cells are scattered throughout.

Studies on tissue engineering of the stomach are very limited. This may be attributed to the unique geometry and biomechanics of the stomach compared to other soft tissues. There are some reports on syngeneic and autologous tissue-engineered stomachs in Lewis rats [8–11] and Yorkshire swine [12], respectively. Although these studies demonstrated a regenerated epithelium organized into gastric glands, epithelial differentiation, and proliferation were not comprehensively analyzed. In addition, investigations involving the mechanism of formation of these tissue-engineered gastric glands are lacking.

Tissue engineering is an interdisciplinary field that combines the knowledge and technology of materials design and optimization, cell cultures, and appropriate use of growth or biochemical factors to create artificial tissues, and regenerate damaged organs [13–22]. Examples of organs that have been tissue engineered include urinary bladder [23], trachea [24, 25], urethra [26], heart [27], liver [28–30], and lung [31, 32].

For tissue engineering, stem cells are derived from a patient, cultured to increase their number, seeded onto a certain carrier or a "scaffold," and then incubated *in vitro* to cellular integration and differentiation. The appropriate factors are also added to the culture system and over a relatively short time, a new tissue is formed. This newly developed tissue is finally ready for implantation to restore the function of a defective organ in a patient [33]. By definition, a scaffold is a three-dimensional (3D) porous construct with pre-tailored architecture and internal morphology that serves as a template for tissue regeneration [34]. It also serves as a carrier for cells, growth factors, or other biomolecular signals. Being porous in nature, a scaffold directs the growth of cells either seeded within its porous structure or migrating from surrounding tissue. Many studies demonstrated the fabrication of scaffolds with different structure and topography.

Electrospinning is a versatile processing technique that can be used for the fabrication of random and aligned fibrous scaffolds with fibers of average diameter in the nm- or μm-scale. The technique utilizes an applied voltage (up to 30 kV) to overcome the surface tension forces of a polymer solution, hence causing it to stretch into fibers that are deposited on a grounded metallic substrate (**Figure 2**) [35, 36]. Various materials have been electrospun into micro- and nano-fibers using electrospinning for a wide range of applications. In tissue engineering, a fibrous scaffold made by electrospinning closely matches the morphology of the extracellular matrix; hence, this increases its potential for tissue regeneration applications. Moreover, the interconnectivity of the pores in 3D-fibrous scaffolds made by electrospinning enhances the communication between the cells in culture, and facilitates their proliferation and differentiation. In contrast, cells grown on 2D platform can proliferate but their differentiation potential would be limited [37].

Various studies developed 3D porous scaffold systems that were either cells-free (acellular) or containing different types of cells. In the acellular approach, cells-free porous scaffolds are implanted inside the internal lining of the stomach to promote the formation of new gastric tissues. Examples included a porous poly(glycolic acid)-reinforced collagen scaffold with a silicone sheet covering the luminal side of the stomach [7, 38]**.** These batches incompletely supported tissue regeneration of the muscular layer and the patch grafts contracted significantly over time. An alternative scaffold composition was developed by Araki et al. and

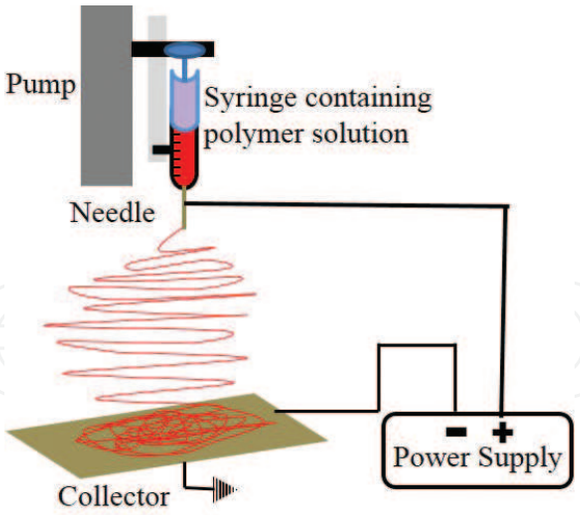

Figure 2. A schematic diagram of the electrospinning process.

was composed of poly(D,L-lactide), poly(ε-caprolactone) (PCL), collagen, and poly(glycolic acid) nonwoven fabric [39]. This multi-layer scaffold was used to repair a large stomach wall defect in a dog without infection or anastomotic dehiscence, and showed sufficient mechanical strength for suturing and better biocompatibility. However, early shrinkage of the implanted scaffold was eventually observed and regeneration of the muscle layer did not occur. In another study, Lourenco et al. developed a model comprising a gastric stromal cell line (NST-20) embedded in a 3D alginate-RGD hydrogel prepared on the basolateral side of a Transwell insert. This assembly closely mimicked the extracellular matrix of the gastric mucosa. It was used for the growth of a moderately differentiated gastric adenocarcinoma cell line (MKN28). This cell-containing scaffold was capable of reproducing the physiological conditions of the gastric barrier [40]. Lourenco et al. proved the closer similarity of this model to the native structure of the gastric mucosa, in which stromal cells appeared to have a role in the establishment of mucosal architecture. This was further confirmed by the production of extracellular matrix. In a different study, isolated gastric epithelial units were seeded onto the inner luminal surface of microporous biodegradable polymer tubes. These tubes were made from a fibrous, nonwoven mesh made of polyglycolic acid and coated with 5% poly-L-lactic acid. The seeded polymer tubes were completely wrapped and sutured into the omentum of adult Lewis rat. These gastric unit/scaffold constructs formed cyst-like structures, which were called "tissue-engineered stomachs" [41]. Recently, Noguchi et al. developed a method to induce the formation of stomach organoids from mouse embryonic stem cells. In this regard, gastric primordial epithelium and underlying mesenchyme were developed using a Matrigel-based 3D culture system. The differentiated organoid were found to contain both corpus- and antrum-specific mature gastric epithelial cells [42]. Finally and in another recent study, scientists grew tissues from the stomach's corpus/fundus region in a petri dish, which were able to produce hydrochloric acid and digestive enzymes [43]. This took place through differentiation of human pluripotent stem cells into gastric organoids containing fundic epithelium [43].

Recently, in our lab, a 3D scaffold made of a biodegradable PCL was fabricated by an electrospinning technique and was used for the growth and differentiation of gastric stem cells [44]. The main objective was to develop the basis for a new modality for the regeneration of defective gastric tissue as a result of gastric cancer or severe gastric ulcer. The experimental procedures and results of this 3D model system are summarized below.

2. Experimental procedures

2.1. Scaffolds preparation and characterization

PCL with an average molecular weight (M_n) of 70,000–90,000 by GPC was used for preparing scaffolds for gastric stem cell culture. An electrospinning technique operating at an applied voltage of 12 kV, a spinning distance of 14 cm, and a feeding rate of 0.16 mL/min, was used in making porous PCL scaffolds. Scaffolds were characterized for their microstructure using a scanning electron microscopy (SEM) technique operating at 15 kV. Tensile properties of the PCL fibrous scaffolds were measured using a universal testing machine MTS with a load cell of 100 kN with an overhead speed of 5 mm/min at room temperature. The measurement of the tensile strength was done in triplicate according to published protocol [45]. For comparison, the stomach wall of 6-month-old mice (n = 3) was used after washing in cold phosphate buffered saline (PBS). Tissues were immediately tested for their tensile strength. Before and after the tensile tests, SEM examination was conducted to investigate the effect of applied load and deformation on the surface topography of the scaffolds.

2.2. Culture of mouse gastric stem (mGS) cells

PCL microporous scaffolds were sterilized with various degrees of ethanol solutions and completely dried prior cell culture experiments. The mGS cells were seeded (2.5×10^5 cells) onto scaffolds (15 mm diameter and 0.9 mm thickness) placed in a 12-well plate, and allowed to grow in a 37°C incubator containing 5% CO_2 and 95% O_2 for up to 12 days. The serum-containing RPMI culture medium was changed every 48 hours. After 3 days in culture, cells were processed for initial screening of cell viability and for microscopic examinations using an inverted Olympus microscope, and Phillips SEM. For SEM, fixation was in 4% paraformaldehyde for 15 min and post-fixation, in 1% osmium tetroxide for 10 min. Following dehydration in ethanol, cells were processed for gold-palladium coating, and finally examined with SEM.

After 3, 6, 9, and 12 days, cultured cells were processed for the quantification of DNA. Cells were washed with PBS and stored at −80°C in 1 ml of Milli-Q water. DNA was extracted from the samples by repeated freeze-thaw cycles followed by ultrasonication. The Quant-iT PicoGreen dsDNA kit was used to quantify DNA according to the manufacturer's instructions. The fluorescence intensity was measured at 520 nm by using the PerkinElmer reader. Scaffolds without cells were used as blank samples. For the measurement of statistical significance, a one way ANOVA with Tukey Multiple Comparison Test was used.

2.3. Immuno- and lectin-cytochemical analysis

The mGS cells cultured on scaffolds for 3 and 9 days were fixed for 15 min in 4% paraformaldehyde. Following PBS wash, cell-containing scaffolds were processed for overnight incubation in 20% buffered sucrose at 4°C. The scaffolds were then mounted on an aluminum stalk using Shandon Cryomatrix. Frozen sections (10–30 micron-thick) were obtained using Cryostome FSE cryostat and immediately mounted on gelatin-coated slides. To visualize cellular morphology and orientation, a few sections were stained with hematoxylin and eosin. The adjacent sections were processed for lectin cytochemistry and immunoprobing of cellular-specific biomarkers.

Following incubation with blocking solution, cryosections were incubated for 60 min with different fluorophore-conjugated lectins: *Ulex europaeus* agglutinin (UEA) I (specific for surface mucous cells), *Griffonia simplicifolia* (GS) II (for mucous neck cells), or *Dolichos biflorus* agglutinin (DBA, for parietal cells) [46, 47]. Cryosections of cell-containing scaffolds were also incubated overnight with several antibodies specific for H,K-ATPase alpha and beta subunits (for parietal cells), TFF1 (for surface mucous cells), TFF2 (for mucous neck), chromogranin-A (for enteroendocrine cells), and ghrelin (for a subtype of enteroendocrine cells). Probed sections were washed in PBS and the appropriate fluorophore-conjugated secondary antibody was added. Finally, cells were visualized using Olympus fluorescence microscope or Nikon confocal microscope.

3. Results and discussion

PCL scaffolds made of microfibers in the range of 2–3 μm in diameter were prepared by electrospinning (**Figure 3**). The scaffold is characterized by a homogeneous fiber size distribution and interconnected porosity. These features are strongly recommended for tissue engineering applications, where the small size fibers provide high surface area for better cell adhesion, while the interconnected porosity provides pathways for cell interaction and new tissue formation. **Figure 4** shows a comparison between the tensile strength of a pure PCL microfibrous scaffold and that of a mouse stomach tissue. On one hand, synthetic scaffold showed a variable degree of tensile strengths at a higher range (0.35–0.6 MPa) than that of natural gastric tissues (0.22 MPa). On the other hand, both synthetic scaffold and natural stomach tissues showed a high degree of elasticity. These properties indicate the suitability of the PCL fibrous scaffold to replace the natural gastric tissues. More importantly, the higher strain of the PCL fibers makes them more durable to expansion and contraction, as dictated by the stomach biomechanical properties. While electrospun PCL fibers appeared nonwoven with random distribution (**Figure 3**), they re-align upon applying tensile forces. This feature is attributed to the interconnected porosity that allows the re-orientation of the fibers during tensile strength measurement.

Microscopic examination of the toluidine blue-stained mGS cells revealed their variable appearance on the PCL microfibrous scaffold [44]. When examined with SEM, they tend to

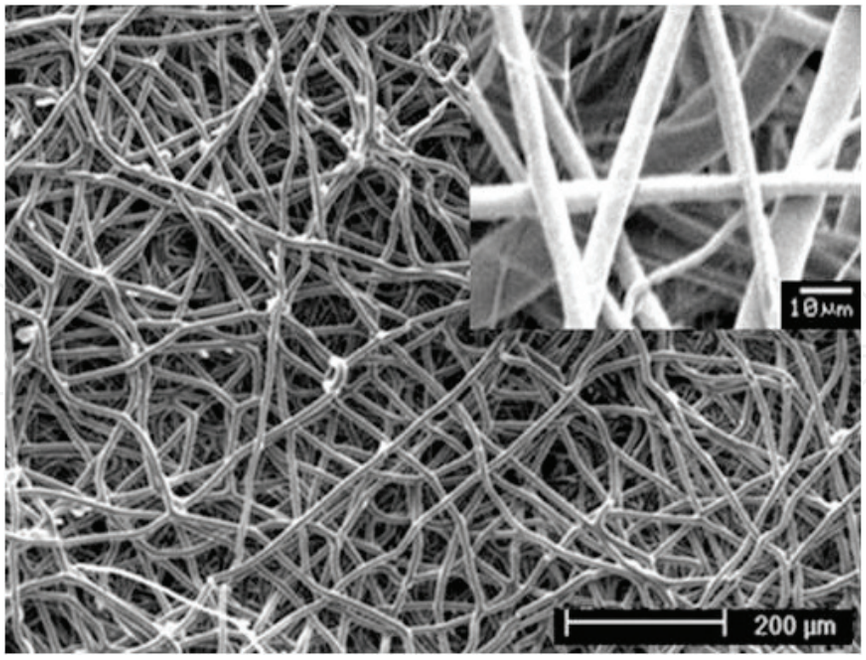

Figure 3. A scanning electron micrograph showing the PCL microfibrous scaffold (insert: a higher magnification of the fibers).

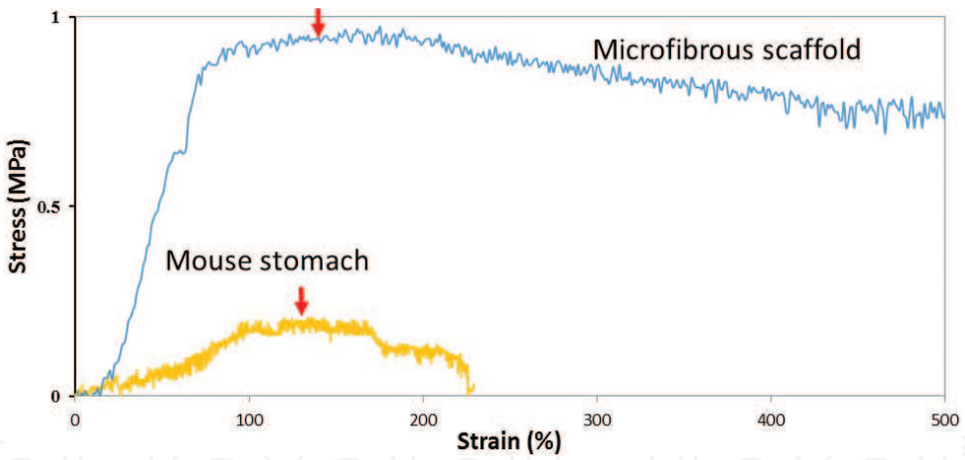

Figure 4. Stress-strain curves showing the tensile strengths of a synthetic PCL microfibrous scaffold (a), and a natural mouse stomach issue (b).

appear flattened [44] with cytoplasmic processes spanning the space between microfibers. Therefore, mGS cells were attached to more than one microfiber and integrated into the pores of the scaffold to grow in 3D (**Figure 5**).

Studies on the surface topography and porosity of scaffolds demonstrated their role on cellular adhesion, growth, and differentiation. Inducing abrasions on the surface of polyvinyl alcohol improved orientation/elongation of fibroblasts and cardiac muscle cells [48]. Different types of scaffolds with variable geometries have been tested for cell culture and adequate growth.

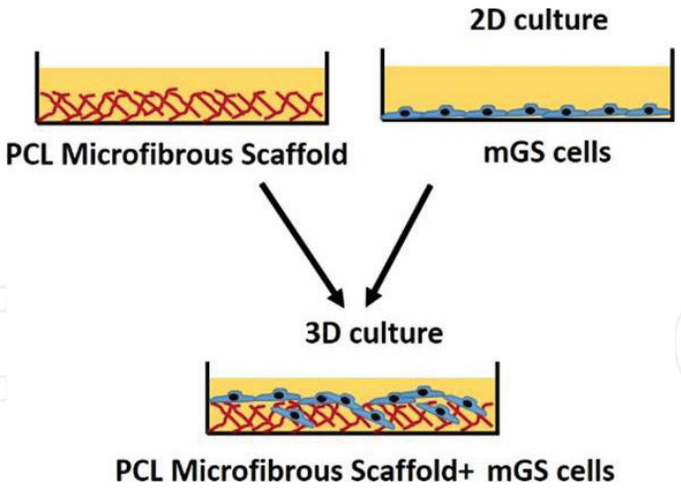

Figure 5. Diagrammatic representation of the microfibrous PCL scaffold immersed in the culture media before and after seeding with mGS cells. Note that the fibrous structure of the scaffold allows the cells to integrate through its interconnected pores and grow in 3D.

The electrospun scaffolds with microfibers seem to be the most suitable because of their 3D architecture, large surface area, and interconnected porosity. It was shown previously that PCL fibrous scaffolds support proliferation and differentiation of mesenchymal stem cells extracted from periodontal ligament [49, 50] and oligodendrocyte precursor cells [51]. Some studies showed that the fiber diameter could influence cell function and behavior on the scaffold [52–57]. Porosity is also an important factor for transport of nutrients and metabolites [58, 59].

Based on the above information, the mechanical and topographical characteristics of the microfibrous scaffold, cell viability and DNA quantification assays were conducted. Both Calcein and MTT cell viability assays showed that microfibrous scaffold support mGS cell growth. The DNA PicoGreen assay was also used to estimate the amount of cells and confirmed the advantage of the fibrous nature of the scaffold in promoting mGS cell binding and growth. It is known that cells interact with the extracellular matrix via integrin binding and sense difference in mechanical stresses through integrin signaling pathway. It was shown that increasing porosity is associated with increasing the expression of integrins [60]. This could partly explain the results obtained in the present study and the value of high porosity of microfibrous scaffold and their significant support to mGS cell growth and attachment.

The mGS cells were seeded on the PCL microfibrous scaffolds for 3, 6, 9, and 12 days to determine their pattern of growth as a function of time. The DNA was extracted from attached cells at different time points and quantified by using PicoGreen assay. The data reflected the number of attached cells on the scaffolds at 3 to 12 days of culture. **Figure 5** in [44] showed that the amount of DNA increased from day 3 to day 6 indicating proliferation of mGS cells on the scaffolds. However, when the cells were cultured for 9 days, the amount of DNA was significantly reduced. A reduction in the amount of DNA was also observed in cells cultured for 12 days [44]. This decrease in the number of cells is either due to down-regulation of cell proliferation or cell death. This effect could be regulated by integrins cytoplasmic domains that are known to affect cell proliferation [61]. Integrin's extracellular domain is also involved in adhesion through interactions with laminin [62]. Targeted deletion of the cytoplasmic domain of integrin

induced reduction in cell proliferation and cell cycle arrest [63, 64]. The microfibers of scaffolds are made of inert material and lack the integrin binding sites. Therefore, the modification in the mGS cell cycle signaling is not expected for mGS cells growing on microfibrous PCL scaffolds. It was interesting to find that the reduction in cell proliferation after 9-day culture was associated with an increase the size of mGS cells [44] which could suggest cell differentiation with even loss of some of the differentiated cells. To test whether the decrease of cell number and the associated cellular enlargement were due to differentiation, cryostat sections of mGS cell-containing scaffolds of 3 and 9 days were processed for antibody probing and lectin binding. Expressions of lineage-specific proteins and glycoconjugates were taken as an indication of cell differentiation. Cryosections stained with hematoxylin and eosin demonstrated general morphology [44]. Adjacent sections probed with anti-TFF2 antibodies revealed that after 9 days of culture of mGS cells on scaffolds, some cells synthesized TFF2 specific for mucous neck cells [65, 44]. When adjacent sections were incubated with a lectin specific for mucous neck cells (GSII), the results revealed positive binding to GSII lectin as seen with fluorescence [44] and confocal [44] microscopes. Therefore, these findings demonstrate that PCL microfibrous scaffolds are suitable for growth and differentiation of mGS cells into mucous neck cells.

4. Conclusions

A synthetic biocompatible microfibrous scaffold made of PCL and fabricated by an electrospinning technique has been used for the culture of mGS cells. The scaffold is characterized by its high surface area and interconnectivity of its 3-dimensional porosity. These factors were shown to provide suitable construct for the proliferation and differentiation of mGS cells. Results showed the continued growth of the mGS cells for 6 days, followed by differentiation at 9 days. Histo- and immunocytochemistry measurements combined with SEM analysis showed multiple evidences in support of the differentiation of the gastric stem cells to mucous neck cells. These results provide the basis for a valid potential application of tissue engineering for regeneration of gastric tissues.

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