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ESTABLISHMENT OF A 3D CULTURE MODEL OF GASTRIC STEM CELLS SUPPORTING THEIR DIFFERENTIATION INTO MUCOUS CELLS USING MICROFIBROUS POLYCAPROLACTONE SCAFFOLD

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United Arab Emirates University
College of Medicine and Health Sciences

ESTABLISHMENT OF A 3D CULTURE MODEL OF GASTRIC STEM
CELLS SUPPORTING THEIR DIFFERENTIATION INTO
MUCOUS CELLS USING MICROFIBROUS
POLYCAPROLACTONE SCAFFOLD

Sunitha Pulikkot

This dissertation is submitted in partial fulfilment of the requirements for the degree
of Doctor of Philosophy

Under the Supervision of Professor Sherif M. Karam

May 2015

Declaration of Original Work

I, Sunitha Pulikkot, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled “*Establishment of a 3D culture model of gastric stem cells supporting their differentiation into mucous cells using microfibrinous polycaprolactone scaffold*”, hereby, solemnly declare that this dissertation is an original research work that has been done and prepared by me under the supervision of Professor Sherif M. Karam, in the College of Medical and Health Sciences at UAEU. This work has not been previously formed as the basis for the award of any academic degree, diploma or a similar title at this or any other university. The materials borrowed from other sources and included in my dissertation have been properly cited and acknowledged.

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Approval of the Doctorate Dissertation


This Doctorate Dissertation is approved by the following Examining Committee Members:

- 1) Advisor (Committee Chair): Sherif M Karam

Title: Professor

Department of Anatomy

College of Medicine and Health Sciences

Signature 

Date May 13, 2015

- 2) Member: Yasir E Griesh

Title: Associate Professor

Department of Chemistry

College of Science

Signature 


Date 13/5/2015

- 3) Member (Internal Examiner): Ernest Adeghate

Title: Professor

Department of Anatomy

College of Medicine and Health Sciences

Signature 

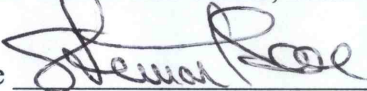
Date 13/5/2015

- 4) Member (External Examiner): Stewart Sell

Title: Professor

College: University at Albany, State University of New York

Institution: Wadsworth Center, State University of New York, USA

Signature 

Date 5/13/15

This Doctorate Dissertation is accepted by:

Dean of the College of Medicine and Health Sciences: Professor Dennis Templeton

Signature _____

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Dean of the College of the Graduate Studies: Professor Nagi T. Wakim

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Abstract

In the stomach, epithelial stem cells are responsible for glandular homeostasis and continuous production of four main cell lineages secreting mucus, acid, pepsinogen and hormones. While alteration in the proliferation and differentiation program of these stem cells is linked to the origin of gastric cancer, they represent an effective target for chemotherapy and a source for cell therapy or tissue engineering in cases of gastric mucosal damage or loss. The aims of this study were 1) to manufacture various forms of scaffolds using a biodegradable polymer (polycaprolactone), 2) to test the suitability of these scaffolds for growth of mouse gastric stem (mGS) cells, and 3) to evaluate whether this culture system could sustain exposure to acidic environment for possible future applications.

Three forms of polycaprolactone scaffold were fabricated: nonporous, microporous and microfibrous. Scanning electron microscopy (SEM) and mechanical testing revealed some similarities between the microfibrous scaffold and extracellular matrix of mouse stomach wall. Examination of mGS cells seeded on different forms of scaffold for 3 days using SEM and calcein viability assay revealed their preferential growth on microfibrous scaffolds fabricated by electrospinning technique.

Analysis of the growth pattern of mGS cells on microfibrous scaffolds following 3, 6, 9 and 12 days of culture using SEM and DNA PicoGreen assay demonstrated an initial increase in cell number, followed by reduction by days 9 and 12. To test whether this reduction was associated with cell differentiation, cryosections of cultured mGS cells on scaffolds were probed with gastric epithelial cell differentiation markers. On day 3, none of the markers bound to the cells. However by day 9, approximately, 50% of the cells bound to N-acetyl-D-

glucosamine-specific lectin (*Griffonia simplicifolia* II) suggesting differentiation into gland mucous cells. This finding was confirmed by the expression of trefoil factor 2 using immunocytochemistry. In addition, gene expression analysis using quantitative reverse transcription polymerase chain reaction (qRT-PCR) demonstrated that the expression of transcription factor SPDEF, required for differentiation of mucous cells, was gradually up-regulated with culture of mGS cells from 3 to 12 days.

To test whether this 3D culture system could tolerate the acidic environment of the stomach, the mechanical/chemical integrity of microfibrinous scaffolds and cultured mGS cells were studied at acidic pH (3.0 to 7.4) using tensile strength measurements, fourier transform infrared spectroscopy, calcein assay, and mRNA/protein expression analysis. The in vitro wound-healing assay was also used to examine effects of acidic pH on cell migration. RPMI culture media at pH 3.0 and 4.5 reduced the mechanical integrity of scaffolds and significantly inhibited cell viability by >70%. However, at pH 5.5 and 6.0, no significant change in cell viability and scaffold integrity was observed, but cell migration was inhibited by more than 50%. Interestingly, only after 3-day culture at pH 5.5, N-acetyl-D-glucosamine-specific lectin binding combined with significant up-regulation in the expression of SPDEF gene confirmed mucous cell differentiation.

In conclusion, a 3D culture model of mGS cells using microfibrinous PCL scaffold supporting their differentiation into gland mucous cells has been established. Reducing the pH value of culture media to 5.5 modulates proliferation/migration programs of mGS cells and speeds up their differentiation into mucous cells. This study provides important basic information for the possible use of mGS cells and microfibrinous PCL scaffolds for future gastric tissue engineering studies and

regenerative therapy of some stomach diseases involving gastric mucosal damage or loss.

Keywords: Stem cells, Cell proliferation, Cell differentiation, Mucous cells, Gastric gland, Stomach, Gastric acid, Tissue engineering, Polycaprolactone, Microfibrous scaffold

Title and Abstract (in Arabic)

تأسيس نموذج ثلاثي الأبعاد لزراعة الخلايا الجذعية للمعدة الداعم لتحويلها إلى خلايا مخاطية باستخدام هياكل ليفية دقيقة من لدائن الكابرولكتون

الملخص

في المعدة تعتبر الخلايا الجذعية مسؤولة عن توازن الغشاء الطلائي ومصدرا أساسيا لأربعة أنواع من الخلايا المفترزة للمخاط، والحمض، انزيم الببسين، والهرمونات ويؤدي التغيير في تكاثر و تمييز هذه الخلايا إلى سرطان المعدة، إلا أن هذه الخلايا تمثل هدفا فعالا للعلاج الكيميائي ومصدرا لعلاج الخلايا أو هندسة الأنسجة في بعض الحالات المرضية للغشاء المخاطي في المعدة. تهدف هذه الدراسة ل(1) تصنيع أشكال مختلفة من الهياكل باستخدام لدائن الكابرولكتون،(2) اختبار مدى فعالية هذه الهياكل لنمو الخلايا الجذعية المعوية للفأر (mGS)، (3) تقييم مدى ثبات هذا النموذج عند تعرضه لبيئة حمضية لتقديم تطبيقات عملية محتملة لهذه الدراسة في المستقبل.

ثلاثة أشكال مختلفة من الهياكل القابلة للتحلل قد تم إنشائها: هياكل غير مسامية، مسامية دقيقة، ليفية دقيقة. و قد كشف المجهر الالكتروني و الاختبارات الميكانيكية عن بعض أوجه التشابه بين الهياكل الليفية الدقيقة و الجدار المعوي للفأر. و عند استخدام المجهر الالكتروني و calcein viability assay لدراسة (mGS) المزروعة على اشكال مختلفة من الهياكل لمدة ثلاثة أيام أن الهياكل الليفية الدقيقة المصنعة بتقنية electrospinning قد اعطت نتائج افضل من غيرها من الهياكل.

وباستخدام المجهر الالكتروني و DNA PicoGreen assay تم ملاحظة زيادة في معدل نمو هذه الخلايا على هياكل الألياف الدقيقة في الايام 3 و 6 من الزراعة، و تلاها بعد ذلك انخفاض في عدد الخلايا و ذلك في اليوم 9 و 12 من الزراعة، و لمعرفة اذا ما كان هذا الانخفاض مرتبط بتمايز الخلايا تم تحضير الخلايا بالتجميد المقطعي تم مراقبة هذه الخلايا باستخدام علامات حيوية خاصة بالتمايز الخلوي للخلايا المعوية. في اليوم الثالث من الزراعة للخلايا لم يتم ملاحظة أي ارتباط مع المؤشرات الحيوية، و لكن في اليوم 9 تم ملاحظة

أن 50% من الخلايا ارتبطت مع *N*-acetyl-D-glucosamine-specific lectin (*Griffonia simplicifolia* II) مشيرة إلى أن هذه الخلايا تتميز لتكوين الخلايا المخاطية. و قد تم اثبات ذلك بالكشف عن الاظهار الجيني ل trefoil factor 2 وذلك باستخدام التحليل الكيميائي الخلوي المناعي. و باستخدام (qRT-PCR) قد تم الكشف ان الاظهار الجيني للعامل SPDEF يعد عاملا اساسيا للتمايز الخلوي للخلايا المخاطية و انه يتزامن مع ارتفاع معدل النمو للخلايا mGS ابتداء من اليوم 3 الى اليوم 12.

و لمعرفة مدى قابلية تحمل الخلايا المزروعة للبيئة الحمضية للمعدة تم اختبار القدرة الميكانيكية و الكيميائية لهياكل الألياف الدقيقة و خلايا mGS في وسط حمضي حيث تبلغ نسبة الحموضة (3.0 to 7.4)، و تم دراسة ذلك باستخدام tensile strength measurements, fourier transform infrared spectroscopy, calcein assay, mRNA/protein expression analysis و إضافة الى ذلك لقد تم استخدام wound-healing assay لدراسة التأثير الحمضي على هجرة الخلايا. لم يتم ملاحظة أي تغيير في التأثير الميكانيكي للهيكل و قدرة الخلايا على النمو في الوسط الحمضي 5.5 و 6.0 لكن تم منع الخلايا المهاجرة بنسبة 50%. و لكن في اليوم الثالث فقط من الزراعة في الوسط الحمضي 5.5 تم ملاحظة وجود ارتفاع في معدل N-acetyl-D-glucosamine-specific lectin binding مع ارتفاع معدل الظهور الجيني ل SPDEF مؤكدا ارتباطه مع التمايز الخلوي للخلايا المخاطية.

إن زراعة الخلايا mGS على هياكل الألياف الدقيقة تدعم التمايز الخلوي للغدة المخاطية وأيضا انخفاض المعدل الحمضي للوسط الزراعي ل 5.5 يغير من معدل النمو و هجرة الخلايا و يسرع معدل التمايز الخلوي لتكوين الخلايا المخاطية . تكمن أهمية هذه الدراسة في توفيرها المعلومات الأساسية لإمكانية استخدام الخلايا الجذعية و هياكل الألياف الدقيقة في هندسة الأنسجة و العلاج التجديدي في علاج بعض الأمراض المعوية التي تشمل حالات القرحة والسرطان للغشاء المخاطي في المعدة.

الكلمات الأساسية:

الخلايا الجذعية، الانتشار الخلوي، التمايز الخلوي، الخلايا المخاطية، الغدة المعوية، المعدة، الحمض المعوي، هندسة الانسجة، هيكل الألياف الدقيقة.

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Almighty Lord and my late father whose abundant blessings gave me strength and confidence to complete my studies.

Dedication

*To my late father who gave me the greatest gift anyone could give another person,
He believed in me.*

Table of Contents

Title	i
Declaration of Original Work	ii
Copyright	iii
Approval of the Doctorate Dissertation	iv
Abstract	vi
Title and Abstract (in Arabic)	ix
Acknowledgements	xi
Dedication	xiii
Table of Contents	xiv
List of Tables.....	xviii
List of Figures	xix
Chapter 1: Introduction	1
1.1 The stomach	1
1.2 The gastric gland	1
1.3 The gastric stem cells	3
1.4 The progeny of gastric stem cells.....	6
1.4.1 Pit cell lineage.....	9
1.4.2 Mucous neck cell lineage.....	9
1.4.3 Zymogenic cell lineage.....	10
1.4.4 Parietal cell lineage.....	10
1.4.5 Enteroendocrine cell lineage.....	11
1.5 Molecular factors underlying gastric stem cell renewal and differentiation	11
1.6 Stem cells and the origin of cancer	19
1.7 Gastric cancer	22
1.8 Tissue engineering	24
1.9 PCL scaffolds	26
1.10 Mouse gastric stem (mGS) cell line	27
1.11 Gastric tissue engineering	28
1.12 Aim of the project	32
Chapter 2: Materials and Methods	33
2.1 Preparation of PCL Scaffolds.....	33

2.2	Scanning electron microscopic (SEM) analysis of scaffolds	33
2.3	Measurement of the tensile strength of the scaffolds using universal mechanical testing machine (MTS).....	36
2.4	Experiment 1: Culture of mGS cells on different PCL scaffolds for 3 days.....	36
2.4.1	Toluidine blue staining for light microscopy.....	37
2.4.2	SEM analysis	37
2.4.3	Cell viability (Calcein assay).....	37
2.4.4	Metabolic activity (MTT assay)	38
2.4.5	Cell quantification using DNA PicoGreen assay.....	38
2.5	Experiment 2: Culture of mGS cells on microfibrinous PCL scaffolds for 3, 6, 9, and 12 days.....	39
2.5.1	Cellular quantitation using DNA PicoGreen assay	39
2.5.2	Gene expression analysis using quantitative reverse transcription polymerase chain reaction (qRT-PCR)	40
2.5.2.1	RNA Extraction	40
2.5.2.2	First Strand cDNA Synthesis	41
2.5.2.3	qRT-PCR	41
2.6	Experiment 3: Culture of mGS cells on microfibrinous PCL scaffolds for 3 and 9 days.....	43
2.6.1	SEM analysis	43
2.6.2	Multi-label immuno- and lectin-cytochemical analysis	43
2.7	Experiment 4: Culture of mGS cells in acidic pH using 2D and 3D systems	45
2.7.1	Effect of acidic pH on the viability of mGS cells in 2D culture	45
2.7.2	Effect of acidic pH on mGS cell migration in 2D culture	46
2.7.3	Effect of acidic pH on microfibrinous PCL scaffolds	46
2.7.3.1	Mechanical testing using MTS.....	47
2.7.3.2	Chemical testing using fourier transform infrared (FTIR) spectroscopy	47
2.7.4	Effect of acidic pH on mGS cells cultured on microfibrinous scaffold	47
2.7.4.1	Cell Viability of mGS cells cultured on scaffold at acidic pH.....	47
2.7.4.2	Quantitative RT-PCR of mGS cells in 2D culture at acidic pH	48
2.7.4.3	Quantitative RT-PCR of mGS cells in 3D culture at acidic pH	48

2.7.4.4 Immuno- and lectin-cytochemistry of mGS cells cultured on microfibrous scaffolds at acidic pH.....	49
Chapter 3: Results	50
3.1 Characterization of PCL Scaffolds.....	50
3.1.1 Morphological Features	50
3.1.2 Mechanical Features	50
3.2 Characterization of mGS cells cultured on PCL scaffolds for 3 days (Experiment 1)	58
3.2.1 Light microscopic features	58
3.2.2 SEM features	60
3.2.3 Cellular viability and quantification	60
3.3 Characterization of mGS cells cultured on microfibrous PCL scaffolds for different time points (Experiment 2).....	66
3.4 Characterization of mGS cells grown on microfibrous PCL scaffolds for 3 and 9 days (Experiment 3)	66
3.4.1 Morphological features	70
3.4.2 Gene expression analysis using qRT-PCR	70
3.4.3 Lectin- and immuno-cytochemical analysis	71
3.5 Effects of acidic pH on cultured mGS cells (Experiment 4).....	75
3.5.1 Effects of acidic pH on the viability of mGS cells in 2D culture	75
3.5.2 Effects of acidic pH on the migration of mGS cells in 2D culture	78
3.5.3 Effects of acidic pH on the mechanical properties of microfibrous PCL scaffolds.....	79
3.5.4 Effects of acidic pH on the chemical properties of microfibrous PCL scaffolds.....	86
3.5.5 Effects of acidic pH on the viability of mGS cells cultured on microfibrous PCL scaffolds.....	86
3.5.6 Effects of acidic pH on gene expression levels of mGS cells seeded in culture plates (2D) and on microfibrous PCL scaffolds (3D)	90
3.5.7 Effects of acidic pH on the lectin- and immuno-cytochemical localization of gastric epithelial biomarkers in mGS cells seeded on microfibrous PCL scaffolds	97
Chapter 4: Discussion	99
4.1 Topographical properties of microfibrous PCL scaffolds suggest their suitability for mGS cell growth	99

4.2	Mechanical properties of microfibrinous PCL scaffolds suggest their suitability for mGS cell growth	101
4.3	Microfibrinous PCL scaffolds are suitable for mGS cell growth.....	102
4.4	Establishment of a three dimensional culture model of mGS cells directing their growth and differentiation into gland mucous cells	107
4.5	Molecular mechanism underlying differentiation of mGS cells into mucous cells.....	109
4.6	Features of mGS cells and PCL scaffolds in acidic environment.....	115
4.6.1	Survival of mGS cells and inhibition of their migration at pH 6.0	115
4.6.2	Microfibrinous PCL scaffolds sustain harsh acidic environment	116
4.6.3	Enhanced expression of mucous cell-specific genes in 3D culture of mGS cells at pH 5.5.....	117
4.6.4	Precocious differentiation of mGS cells into mucous cells	119
Chapter 5: Conclusion.....		122
Bibliography.....		124

List of Tables

Table 1: Factors influencing gastric epithelial cell proliferation and differentiation	14
Table 2: List of gene-specific primers used for quantitative RT-PCR studies	42
Table 3: Tensile performance (stress and strain) of different PCL scaffolds as compared to mouse stomach tissue.....	59

List of Figures

Figure 1: Diagrams depicting the structure of the stomach and gastric gland.	2
Figure 2: Fate map of gastric stem cells.....	8
Figure 3: Summary diagram of the preparation of 3 different types of PCL scaffolds (nonporous, microporous, and microfibrous) and their use in mGS cell culture for different time points and assays.	34
Figure 4: Diagram representing the process of electrospinning.....	35
Figure 5: SEM micrographs of nonporous (a), microporous (b) and microfibrous (c,d) scaffolds showing their surface topography.	51
Figure 6: Representative samples of nonporous (a, b) and microfibrous (c, d) PCL scaffolds before (a, c) and after (b, d) tensile testing.	53
Figure 7: The mouse stomach wall (a, b) and microfibrous PCL scaffold (c, d) samples as they appear before (a, c) and after (b) or during (d) tensile testing.	54
Figure 8: SEM images of microfibrous PCL scaffold samples before (a) and after (b) conducting the tensile test.	55
Figure 9: Stress-strain curves of nonporous (a), microporous (b), and microfibrous (c) PCL scaffold samples and also for the mouse stomach wall (d).	57
Figure 10: Lectin cytochemistry for the mGS cells cultured on coverslips. Fluorescence micrographs show the blue nuclear staining with DAPI (a, b, c, d) and the binding of WGA (green) (a).....	62
Figure 11: Light micrographs of toluidine blue-stained mGS cells after 3 days culture on the surfaces of nonporous (a), microporous (b), and microfibrous (c) PCL scaffolds.....	63
Figure 12: Scanning electron micrographs of mGS cells cultured on nonporous (a), microporous (b), and microfibrous (c) PCL scaffolds for 3 days.....	64

Figure 13: Cell viability assay for mGS cells after 3 days of culture on different types of scaffolds: nonporous (NPS), microporous (MPS) and microfibrinous (MFS).	65
Figure 14: Cell metabolic activity assay using MTT reagent for mGS cells after 3 days of culture on different types of polycaprolactone scaffolds: nonporous (NPS), microporous (MPS) and microfibrinous (MFS).....	67
Figure 15: DNA PicoGreen assay for quantification of unattached mGS cells after 3 days of culture on 3 types of scaffolds: nonporous (NPS), microporous (MPS) and microfibrinous (MFS).....	68
Figure 16: Estimation of DNA content of mouse gastric stem cells cultured on microfibrinous polycaprolactone scaffolds for 3, 6, 9 and 12 days using PicoGreen assay.	69
Figure 17: Scanning electron micrograph of mouse gastric stem cells cultured on microfibrinous polycaprolactone scaffolds for 3 (a) and 9 (b) days.	72
Figure 18: mRNA expression of Oct4 (a), DCLK1 (b), and PCNA (c) in mGS cells grown on culture plate (control) and on microfibrinous scaffolds for 3, 6, 9, and 12 days and normalised with GAPDH.	73
Figure 19: Estimation of SPDEF (a) and XBP1 (b) mRNA expression in the mGS cells grown on microfibrinous scaffold for 3, 6, 9, and 12 days normalised with GAPDH expression.....	74
Figure 20: Microscopic analysis of cryostat section of mGS cells growing on microfibrinous PCL scaffolds for 9 days.	76
Figure 21: Lectin histochemical analysis for cryosections of mGS cells growing on microfibrinous scaffolds for 9 days.	77
Figure 22: Cell viability and death assay of mGS cells cultured in RPMI at different pH values and incubated with calcein (a) and propidium iodide (b).....	80
Figure 23: Fluorescence micrographs of calcein (a,c,e,g) and double calcein-propidium iodide (b,d,f,h) labeling of mGS cells cultured for 2 days in	

normal RPMI and then for 5 hours in RPMI media at pH values of 3.0 (a, b), 4.5 (c, d), 5.5 (e, f), and 7.4 (g, h).	81
Figure 24: Phase contrast microscopic images of wounded monolayers of mGS cells incubated in RPMI media at pH 7.4 (a,c,e,g) and 6.0 (b,d,f,h) for 1hr (a, b), 1day (c, d), 2 days (e, f), and 3 days (g,h).....	82
Figure 25: In vitro wound healing assay.	83
Figure 26: Scanning electron micrographs of microfibrinous PCL scaffolds showing their surface topography at low (a) and high (b) magnifications. (a)	84
Figure 27: Measurements of stress of the microfibrinous PCL scaffolds incubated for 3, 6, 9, and 12 days in RPMI media at pH 3, 5.5, and 7.4.....	85
Figure 28: Infrared spectroscopy analysis of untreated PCL microfibrinous scaffold (a) and PCL microfibrinous scaffold samples incubated at the pH 3.0 (b), 5.5 (c) and 7.4 (d) for 12 days.....	87
Figure 29: Fluorescence micrographs of calcein (a, c, e) and calcein plus propidium iodide (b, d, f) labeling of mGS cells grown on microfibrinous PCL scaffolds for 2 days in RPMI media at pH 7.4 and then for 3 hours in RPMI media of pH 3.0 (a, b), 5.5 (c, d) and 7.4 (e, f).	88
Figure 30: Cell viability and death assay of mouse gastric stem cells grown on microfibrinous PCL scaffolds using RPMI at pH values of 3.0, 5.5, and 7.4.....	89
Figure 31: The mRNA expression of DCLK1 in mGS cells grown in 2D culture plates at pH 5.5 for 3 and 9 days as compared to control cells growing at pH 7.4.....	91
Figure 32: The mRNA expression of PCNA in mGS cells grown in 2D culture at pH 5.5 for 3 and 9 days.....	92
Figure 33: The expression of DCLK1 mRNA in mGS cells grown in 3D culture incubated at pH 5.5 and 7.4 for 3 and 9 days.....	93

Figure 34: The mRNA expression of PCNA in mGS cells grown on 3D (PCL microfibrinous scaffold) cell culture incubated at pH 5.5 and 7.4 for 3 and 9 days.	94
Figure 35: Expression of the mRNA of the transcription factor SPDEF in mGS cells grown on 3D (microfibrinous) PCL scaffolds and incubated at pH 5.5 and 7.4 for 3 days.....	95
Figure 36: Expression of the mRNA of the transcription factor XBP1 in mGS cells grown on 3D (microfibrinous) PCL scaffolds and incubated at pH 5.5 and 7.4 for 3 days.....	96
Figure 37: Fluorescence micrographs of mGS cells growing on microfibrinous PCL scaffolds for 3 days using RPMI media at pH values of 7.4 (a) and 5.5 (b) and probed with DAPI (blue) and GSII (green).	98
Figure 38: Comparison of the tensile curves of nonporous, microporous, and microfibrinous PCL scaffold samples (a) and comparison of the tensile curves of the mouse stomach wall with the microfibrinous samples (b)	103
Figure 39: Diagrammatic representation of mGS cell growth on nonporous, microporous, and microfibrinous PCL scaffolds for 3 days.	104
Figure 40: Diagram representing the differentiation of gastric stem cell into gland mucous cell after 9-day-culture on microfibrinous PCL scaffold and the changes that occur in the expression pattern of genes involved.	114
Figure 41: Diagram representing the differentiation of gastric stem cell into gland mucous cell after 9-day-culture in acidic pH on microfibrinous PCL scaffold and the changes that occur in the expression pattern of genes involved.....	121

Chapter 1: Introduction

1.1 The stomach

The stomach is the most dilated part of the digestive tube which connects the esophagus with the small intestine (Fig. 1). The shape and position of stomach are highly variable due to several factors such as the amount of food content, the process of digestion, and the degree of the development of the gastric musculature. The wall of the stomach comprises four coats; serosa, muscularis, submucosa, and mucosa. The serosa is the outermost layer and represents the peritoneal covering of the stomach. The muscularis is made of smooth muscle fibers. The submucosal layer consists of a loose tissue connecting the mucosa and muscularis layers. The mucosa is the innermost layer and includes numerous tubular glands (Fig. 1).

1.2 The gastric gland

The luminal surface of the stomach has little indentations known as gastric pits (foveolae) representing the openings of gastric glands that extend deep in the mucosa. The gastric glands in the cardiac and pyloric portions of the stomach are mostly populated by mucous cells and enteroendocrine cells. The gastric gland in the corpus region is made of 4 regions: pit, isthmus, neck, and base (Fig.1b). The pit and neck regions are populated by different mucous cells. In the base, the pepsinogen-secreting chief or zymogenic cells predominate. Both the acid-secreting parietal cells and hormone-secreting enteroendocrine cells are scattered throughout the 4 gland regions.

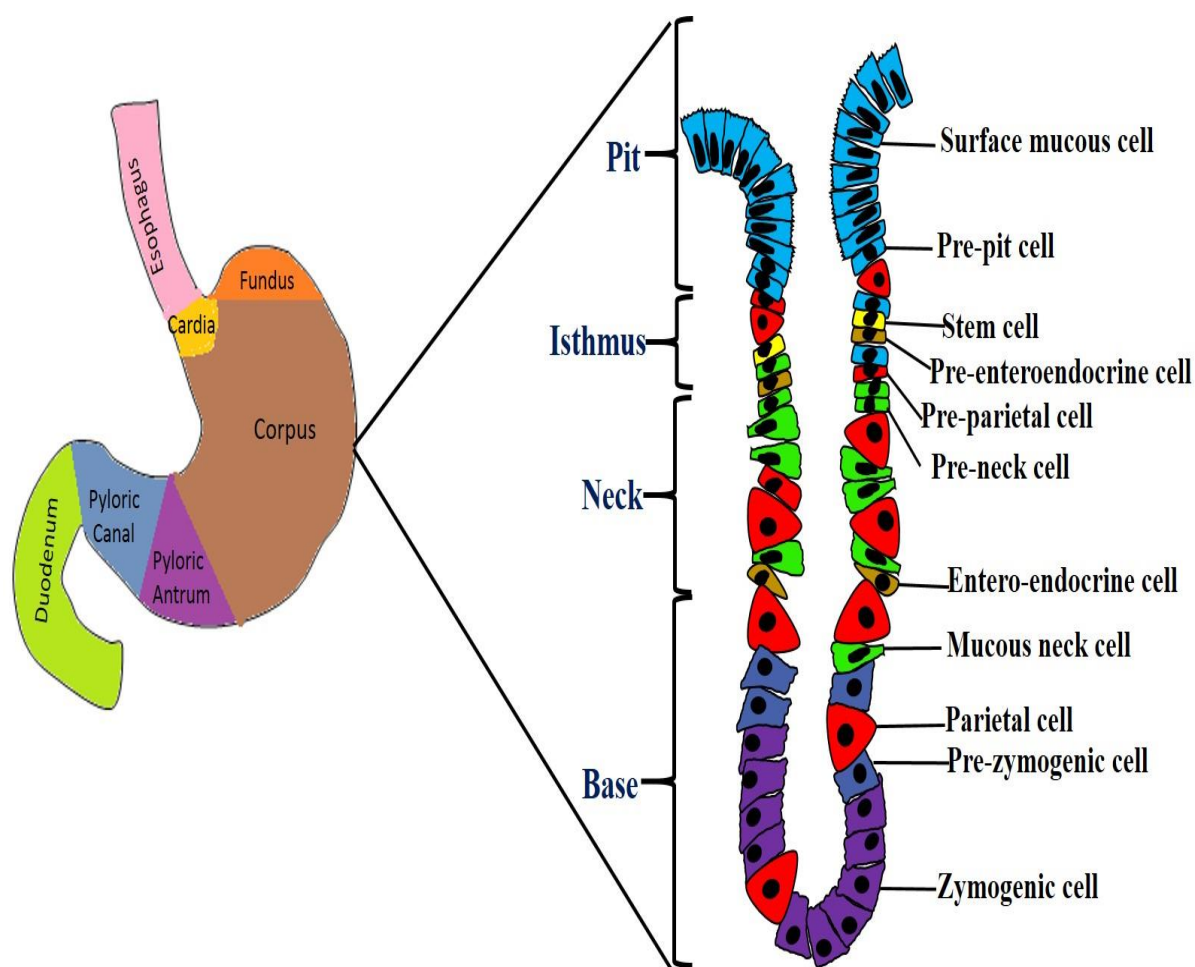


Figure 1: Diagrams depicting the structure of the stomach and gastric gland. The stomach is connected to the esophagus cranially and the duodenum caudally. The stomach includes the cardia, fundus, corpus, and pyloric antrum/canal. The gastric gland comprises 4 regions: pit, isthmus, neck and base. They are respectively populated by surface mucous cells, progenitor/stem cells, mucous neck cells, and zymogenic cells. Both parietal and enteroendocrine cells can be found in any of the 4 gland regions. The progenitor cells of the isthmus include pre-pit, pre-neck, pre-parietal and pre-enteroendocrine cells.

1.3 The gastric stem cells

In mice, the gut epithelium is first identifiable at embryonic day 7 as a single layer of proliferative endodermal cells (Maunoury et al., 1992). Then, within few days the endoderm forms pseudostratified epithelium followed by elongation of the gut tube and its compartmentalization with remarkable changes in the lining epithelium (Karam, 1999). By using electron microscopy and ³H-thymidine radioautography, undifferentiated granule-free stem cells located in the isthmus region of the gastric gland were identified (Karam & Leblond, 1993a). In the corpus region of the adult stomach, the stem cells are found at the junction between the pit region and the neck of the gastric gland in a narrow zone referred to as “isthmus” (Karam & Leblond, 1992). These isthmal cells actively divide to maintain themselves and to produce committed progenitors that undergo differentiation and give rise to specialized cells. Differentiation of isthmal progenitor cells is associated with their migration in a bipolar fashion (Karam, 1993; Karam & Leblond, 1993a-d).

In the late 1940s, Leblond et al identified the location of ³²P-labeled nucleotides that were incorporated into nuclei of live cells. In the stomach, radio-labeled cells appeared just below the pits or foveolae, the microscopic openings of gastric gland units into the stomach lumen. The investigators concluded that this region of anatomic narrowing, the isthmus, was the site of cellular renewal in undamaged tissue.

In 1953, Stevens and Leblond made the observation that mucous cells lining the gastric lumen normally undergo continuous renewal. With the advent of ³H-thymidine radioautography, it became possible to visualize the migration of these cells along the pit wall. In 1966, Richard Corpron analyzed his own findings with those from the few available ultra-structural studies of the rat gastric corpus and concluded

that “nondifferentiated cells” in the isthmus were the source of all other mucosal cells. Although Corpron did not use the term “stem cell,” he did localize and identify cells with undifferentiated morphology as the probable origin of all other epithelial cells. Light and electron microscopy methods combined with radioautography revealed that other gastric epithelial cells also undergo continuous renewal (Karam & Leblond, 1993a-d).

At birth, the gastric glands in both the corpus and pylorus are polyclonal. In contrast, during adulthood, X chromosome inactivation and chemical mutagenesis studies have shown that 90-95% of the gastric glands in the pylorus and corpus regions become monoclonal (Nomura et al., 1998; Tatematsu et al., 1994). This indicates that each gastric gland is derived from a single multipotent stem cell.

In 2002, Bjerknes and Cheng provided an additional functional evidence for the existence of these multipotent stem cells in the oxyntic region of the adult mouse stomach. The authors took advantage of the ubiquitous expression of LacZ allele in the ROSA26 LacZ mice to induce, by chemical random mutagenesis, a loss of gene expression at low frequency in the gastric epithelium of adult hemizygous mice. At later time points, LacZ negative clones within the epithelium were found to contain all four major gastric cell lineages, consistent with the notion that they are derived from a common precursor, the multipotent stem cell. Since the initial mutation event leading to loss of reporter gene activity in this model occurred at random, the identity of the stem cell was not revealed (Bjerknes & Cheng, 2002). However, the identity of these cells was defined in the earlier study as undifferentiated granule-free cell in the isthmus of the oxyntic units (Karam & Leblond, 1993a). These stem cells were the most proliferative and ultra-structurally characterized by a high nucleus-to-cytoplasm ratio, a lack of secretory granules, few small mitochondria and many free ribosomes.

Using these morphological criteria, the corpus granule-free stem cell population was isolated using laser capture micro dissection and the genetic profile of these cells was defined. Gastric stem cell profiling revealed high expression levels of genes regulating signaling pathway of insulin-like growth factor, proteosomal degradation, RNA processing and localization, as well as genes involved in the Wnt signaling pathways. Indeed, this genetic profile resembles that of the embryonic stem cells, highlighting the immature/progenitor nature of granule-free cells (Giannakis et al., 2006; Mills et al., 2002).

There is another group of cells serving as reserve stem cells and called differentiated *Troy*⁺ chief cells. They were induced by the depletion of the proliferating cells in the isthmus compartment in the corpus region. *Troy* potentially functions as a receptor for lymphotoxin A. This subpopulation of chief cells share chief cell markers like *Gif*, *Mist* as well as Wnt driven stem cell markers such as *Axin 2*, *Ephb2* and *CD44* and able to drive the differentiation towards mucous neck cells and pit cell lineages (Stange et al., 2013).

In the isthmus of the antro-pyloric glands, the existence of undifferentiated mottled-granule cells were found to undergo clonal expansion and give rise to two types of progenitor cells: dense-granule cells (pit cell progenitors) and core granule cells (gland cell progenitors) which give rise to mucus-secreting pit and gland cells (Lee & Leblond, 1985). Therefore, in both oxyntic and pyloric antral regions, the stem cells located in the isthmus proliferate and their immediate progeny differentiate within the isthmus while migrating bi-directionally towards the pit and the gland regions (Lee & Leblond, 1985; Karam & Leblond, 1993a).

Recently, Hans Clevers' group showed that the stem cell marker, *Lgr5* is expressed in a specific population of cells located at the very bottom of the pyloric

gastric glands (Barker et al., 2010; Leushacke et al., 2013). The use of transmission electron microscopy combined with cryo-immuno gold labelling showed that Lgr5 cells represent classical features of immature cells such as limited basal rough endoplasmic reticulum, a large centrally located nucleus, and apical microvilli. More mature cells with abundant apical granules occupied the positions just above the Lgr5 cell zone. Lgr5 cells were absent from the isthmus region of the pyloric glands, where the mottled-granule cells are located.

To test the stemness of these Lgr5 expressing cells in the stomach antrum, lineage tracing experiments were conducted in Lgr5-EGFP-IRES-CreERT2/Rosa26R LacZ reporter mice. This study demonstrated that Lgr5 expressing cells were cycling adult stem cells and able to produce the different cell lineages of the antro-pyloric units and therefore, are considered multipotent stem cells. The genetic profile of these cells is characterized by the expression of several Wnt target genes, whereas differentiated endocrine or mucin expressing genes are absent (Vries et al., 2010).

1.4 The progeny of gastric stem cells

The stem cells of the stomach are stationary anchored in specific location (isthmus region) where decisions concerning proliferation and differentiation/migration pathways are made. The turnover times of the isthmal stem cells of the oxyntic gland and pyloric antral gland are about 2.5 days and 1 day, respectively (Karam & Leblond, 1993a; Lee & Leblond, 1985).

According to their distribution in the 4 successive glandular regions (pit, isthmus, neck, and base), the self-renewing epithelium of the stomach body contains 11 different types of cells: gastric stem cells, pre-pit cells, pit cells, pre-parietal cells, parietal cells, pre-neck cells, mucous neck cells, pre-zymogenic cells, zymogenic

cells, pre-enteroendocrine cells and enteroendocrine cells (Karam & Leblond, 1992). The stem cells reside in the isthmus region and give rise to four types of terminally differentiated cells that are replaced at different rates: oxyntic (parietal) cells, zymogenic (chief) cells, surface mucous (foveolar or pit) cells, and enteroendocrine cells (Fig.2). Mucous neck cells function as secretory cells and as intermediate progenitors for chief cells. Around 19.1% surface mucous cells, 6.5% mucous neck cells, 34.7% zymogenic cells, 13.4% parietal cells and 6.8% entero-endocrine cells comprises the gastric gland of corpus mucosa (Karam & Leblond, 1992). In the stomach, the pit, parietal and zymogenic cells have different turnover times: 3, 54 and 194 days, respectively.

The mucous glands of the pyloric antrum are populated by pit cells which migrate outwards and gland cells which migrate inwards; their turnover times are about 3 and 1-60 days respectively (Lee & Leblond, 1985). The isthmus cells give rise to both pit cell and gland cell lineages. Pre-pit cells accounts for 17% of all isthmus cells located near the pit border have the same morphological features and dynamic behavior of the pre-pit cells in the oxyntic epithelium. Pit cells represent about 180 cells per gland and are located in the pit region. Poorly differentiated pre-gland cells represent about 28% of the isthmus cells predominate in the neck border. They duplicate, differentiate, and migrate to cross the neck border and become gland cells, which accounts for 37 cells per unit (Lee & Leblond, 1985).

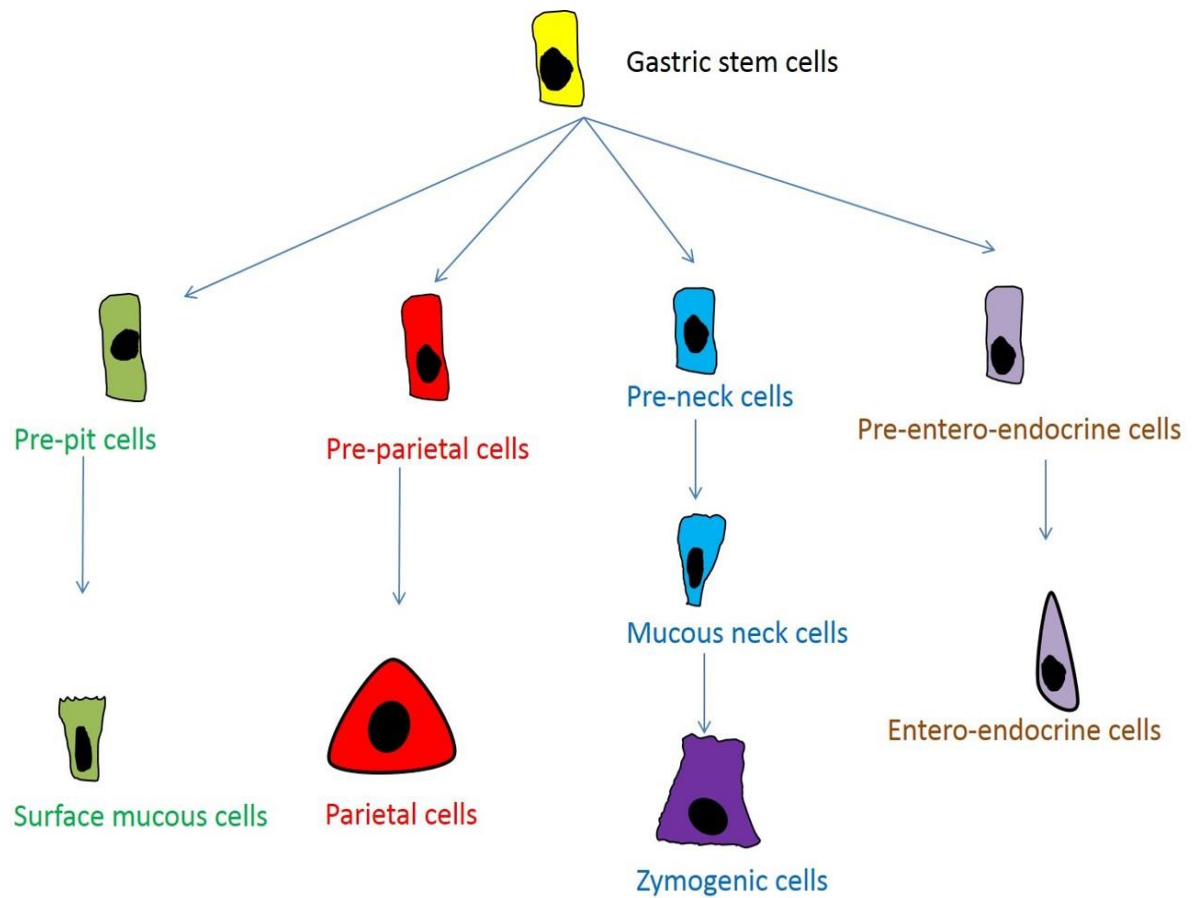


Figure 2: Fate map of gastric stem cells. In the isthmus region of the glandular epithelium of the gastric corpus, the stem cells give rise to 4 main progenitors: pre-pit, pre-parietal, pre-neck, and pre-enteroendocrine cells. These progenitors differentiate while migrating away from the isthmus and give rise to surface mucous, parietal, mucous neck, zymogenic and enteroendocrine cells.

1.4.1 Pit cell lineage

Stem cells differentiate and migrate upward and the 67% of their progeny become pre-pit cell precursors. These precursors are characterized by the presence of small Golgi apparatus and apparently thought to produce a progeny of two types: pre-pit cells and pre-parietal cells with pre-pit cell like secretory granules. Pre-pit cells localized on the upper segment of the isthmus are characterized by 200nm wide secretory granules located in the Golgi region. Pre-pit cells migrate outward along the pit wall and mature to form pit cells or surface mucous cells. It takes about 60 hr. to reach the surface. The secretory vesicles increases in size around 400nm in the pit region (Karam & Leblond, 1993b) whereas at the surface, the cells activity diminishes which is clear by the overall reduction in the nucleoli and mitochondrial size, lysosomal body formation which ultimately results in cell death. The overall turnover time of pit cells averages 3 days.

1.4.2 Mucous neck cell lineage

The stem cells differentiate and move downwards and around 24% gives rise to pre-neck cell precursors characterized by prosecretory vesicles at the trans-face of their Golgi apparatus containing dense irregular material with light periphery. Few pre-parietal cells are also produced with the secretory granules similar to those of pre-neck cells. Pre-neck cells are located in the lower portion of the isthmus and have few 400nm wide secretory granules which appear dense with a light core. Pre-neck cells have a turnover time of 3 days (Karam & Leblond, 1993c) mature to form mucous neck cells which contain many dense mucous granules with light core made up of pepsinogen (Sato & Spicer, 1980). Mucous neck cells near the isthmus have 430nm secretory granules. As they migrate to the base region, the granule size

increases to 700nm. The life span of these intermediate cells are 7 to 14 day. By this time, their phenotype gradually changes from mucous to serous (Karam & Leblond, 1993c).

1.4.3 Zymogenic cell lineage

Mucous neck cells are responsible for the development of zymogenic cell lineage. Pre-zymogenic cells developed from the mucous cells exhibit more endoplasmic reticulum cisternae and gradual change in their secretory granules. As these intermediate cells migrate downward, they produce secretory granules which become more and more pepsinogenic. The zymogenic cells are pepsinogen-secreting cells and their granule size varies from 780 to 1070 nm. Zymogenic cells are characterized by large amount of rough ER cisternae and the enlarged nucleolus. The turnover time of zymogenic cells is around 194 days (Karam & Leblond, 1993c).

1.4.4 Parietal cell lineage

Parietal cells are produced in the isthmus and migrate bi-directionally along the gland axis. Parietal cells are the mature form of cells developed from the pre-parietal cells. Pre-parietal cells are characterized by embryonic cell-like features, in addition they have numerous apical microvilli with little glycocalyx. While its development, pre-parietal cells acquire many changes such as a few small H,K-ATPase-containing tubules and vesicles, incipient canaliculus, and increase in the number and size of mitochondria. Expansion of the canaliculi and overall increase in cell size are associated with the formation of a fully mature parietal cell. The overall development of a parietal cell requires 2 or 3 days. The estimated turnover time of parietal cells is about 54 days (Karam, 1993; Karam & Forte, 1994).

1.4.5 Enteroendocrine cell lineage

Endocrine cells are the hormone-secreting cells. Despite expressing a common set of genes, neurons and endocrine cells have different embryological origin. Stem cells give rise to pre-enteroendocrine cells which carry enteroendocrine type of secretory granules. These immature cells produce mature forms enteroendocrine cells which reside in all four regions of the gastric gland; they are less frequent in the pit, intermediate in the neck and isthmus and frequent in the base region (Karam & Leblond, 1992; 1993d). In mice there are many types of enteroendocrine cells which are named according to the types of hormones they secrete, such as G (producing gastrin), D (somatostatin), A (glucagon), EC (serotonin), ECL (histamine) and ghrelin cells.

1.5 Molecular factors underlying gastric stem cell renewal and differentiation

In vertebrates, the development of the digestive tract starts from an undifferentiated simple tube which rostro-caudally divided into esophagus, stomach, small intestine, caecum and large intestine. All these organs contain an epithelial lining originated from endoderm and as surrounding mesenchyme developed from a splanchnic mesoderm (Romanoff et al., 1960). All these regions have different histological architecture as well as gene expression profiles leading to different functions such as digestion, absorption, and excretion. During organogenesis, the interaction between epithelium and mesenchyme is crucial. It has been shown in mouse and chicken models through tissue grafting experiments that the source of mesenchyme is important for gut endoderm differentiation (Kedinger et al., 1986; Mizuno & Yasugi, 1990).

Factors influencing gastric stem cell proliferation and differentiation can be categorized into transcription factors, signaling molecules, hormones & cytokines, receptors and others; some of these factors are summarized in table 1.

Continuous self-renewal of gastric stem cells and formation of various differentiated epithelial cells are very well regulated in the gastric glands. Their proliferation and potential to form the whole gland at the time of injury or regeneration has been demonstrated in various models. But their interactions with other signaling cascades were not well documented. For example, Sox2 is one the pluripotent marker expressed in gastric stem cells. Sox2 expressing cells were able to give rise to all the other stomach lineages (Arnold et al., 2011). It was reported recently in a mouse model lacking *Agr2* that the mucous neck cells were hyperproliferated expressing *sox9* and the production of parietal and zymogenic cells was down-regulated (Gupta et al., 2013). Similarly it was reported in 2012 that the Oct4 upregulation was associated with carcinogenesis where as in normal gastric tissues Oct4 were present in GSII and UEA stained cells (Al-Marzoqee et al., 2012). Doublecortin and calcium/calmodulin-dependent protein kinase-like-1 (DCLK1) is a candidate marker for progenitor cells in the gastrointestinal mucosa. The tubulin binding part of the protein is involved in shaping the cytoskeleton, thereby regulating cell motility and axonal migration as well as differentiation and the cell cycle while the protein kinase function is unknown. Inhibition of notch signaling reduces the DCLKI-expressing stem cell number (Qu et al., 2014). However, the stem cell nature of DCLK1-expressing cells is questioned. DCLK1 is a specific marker of tuft or caveolated cells (Gerbe et al., 2009). Gerbe and colleagues studied DCLK-1 positive cells in mouse small intestine and demonstrated that they are secretory cells expressing COX-1, COX-2 and β -endorphin (Gerbe et al., 2011). Sox 2 along with

other transcription factors possessed the ability to reprogram differentiated adult cells to a state of pluripotency, resembling that seen in embryonic stem cells (Aoi et al., 2008; Takahashi et al., 2007; Takahashi & Yamanaka, 2006). Sox17 is expressed in the esophagus and stomach and Sox18 is expressed only in the stomach. Sox 2 expression is markedly down-regulated in gastric carcinomas indicating aberrant expression of the gene with a loss of proper cellular homeostasis (Li et al., 2004; Que et al., 2007; Sanada et al., 2006). It has been shown recently that Sox17 acts in combination with others factors like Hex1 and Pdx1 to specify different organ lineages from a common pool of progenitor cells in ventral foregut (Spence et al., 2009). Also, Sox2 is found to upregulate the expression of pepsinogen A in gastric cell lines. This has been confirmed when the interference in Sox2 expression results in decrease of the expression of pepsinogen A (Tani et al., 2007). A detailed understanding of the regulations of the different differentiation program will be essential for understanding of the basic biology of gastric stem cells and their possible role in cancer and regeneration of damaged gastric epithelium.

Table 1: Factors influencing gastric epithelial cell proliferation and differentiation

FACTORS	Functions or Cell types	References
Transcription factors		
Mist 1	Zymogenic cells	(Huh et al., 2010; Ramsey et al., 2007; Tian et al., 2010)
Sall4	Fetal gut differentiation	(Ushiku et al., 2010)
Pdx-1	G cells	(Larsson et al., 1996)
Myc	Cell proliferation	(Larsson et al., 1996)
Hes-1	Enteroendocrine cells	(Jensen et al., 2000)
Akt	Cell proliferation	(Sasaki et al., 2013)
GATA-6	Endocrine cells	(Dimaline et al., 1997)
GATA-4	Cytodifferentiation, Parietal cells	(Jacobsen et al., 2002a, 2005)
Runx3	Chief cells	(Ito et al., 2011; Ogasawara et al., 2009)
Ngn3	Endocrine cells	(Jenny et al., 2002; Lee et al., 2002)
Nkx 6.3	G cells	(Choi et al., 2008)
Pax 4&6	Endocrine cells	(Larsson et al., 1998)
Sox-2	Surface Mucous cells	(Que et al., 2007)
GATA-5	Gland mucous cells	(Sakamoto et al., 2000)
XBP1	Zymogenic cells	(Huh et al., 2010)
Rab 3d	Zymogenic cells	(Tian et al., 2010)
Rab 26	Zymogenic cells	(Tian et al., 2010)
FOXQ1	Surface mucous cells	(Verzi et al., 2008)
Mash 1	Endocrine cells	(Kokubu et al., 2008)
Spdef	Mucous gland cells	(Horst et al., 2010)
Agr2	Surface mucous cells, Mucous neck cells, Enteroendocrine cells	(Gupta et al., 2013)
Nkx 2.2	G cells	(Desai et al., 2008)
Arx	G cells	(Du et al., 2012)
Signaling Molecules		
Wnt	Parietal cell maturation	(Jain et al., 2006; Radulescu et al., 2013)
Reg1	Parietal cells, Zymogenic cells, Cell proliferation	(Kinoshita et al., 2004; Miyaoka et al., 2004)
BMP2	Surface mucous cells, Cell proliferation	(Itoh et al., 2006; J. Zhang et al., 2012)
BMP4	Parietal cells	(Nitsche et al., 2007)
BMP7	Cell proliferation	(Aoki et al., 2011)
BMP	Enteroendocrine cells	(Maloum et al., 2011)
TGF- α	Mucosal cells	(Chen et al., 1993; Coffey et al., 1995; Rutten et al., 1993)
IHH	Pit cells	(Fukaya et al., 2006)

SHH	Parietal cells, Surface mucous cells, Zymogenic cells	(van den Brink et al., 2001; Kim & Shivdasani, 2011; Stepan et al., 2005; Tanaka et al., 2014)
Notch	Enteroendocrine cells, Cell proliferation	(Bredemeyer et al., 2009; Jensen et al., 2000)
cd2ap	Cell motility	(Karam et al., 2005)
TFF1	Surface mucous cells, Cell proliferation	(Karam, 2008; Tomita et al., 2011)
Activin	Surface mucous cells, Mucous neck cells, Parietal cells	(Li et al., 1998)
Growth Factors		
EGF	Parietal cells Surface mucous cells	(Coffey et al., 1995; Ichikawa et al., 2000; Rutten et al., 1993)
Retinoic acid	Zymogenic cells, Cell proliferation	(Karam et al., 2005)
Huntingtin-interacting protein 1	Zymogenic cells, Parietal cells	(Keeley & Samuelson, 2010; Liu et al., 2012)
FGF-10	Endocrine cells, Parietal cells, Cell proliferation	(Nyeng et al., 2007; Ohning et al., 1996; Shin et al., 2006; Spencer-Dene et al., 2006)
Hepatocyte growth factor	Cell proliferation	(Yamagata et al., 2012)
Hormone & Cytokines		
Gastrin	Parietal cells, Mucosal cells, ECL cells, Cell proliferation	(Jain et al., 2006; Kidd et al., 2000; Tomita et al., 2011; Walsh, 1988; Walsh & Grossman, 1975a, 1975b; Wang et al., 1996)
TGF- α	Mucous neck cells, Cell proliferation	(Dempsey et al., 1992; Kobayashi et al., 2000; Osaki et al., 2010)
Histamine	ECL cells	(Fiorucci et al., 1996; Kobayashi et al., 2000; Ogawa et al., 2003; Tanaka et al., 2002)
TNF- α	Zymogenic cells	(Fiorucci et al., 1996)
Interleukin-1 β	Proliferation	(El-Omar et al., 2000; Kato et al., 1999; Tanaka et al., 2014)
Ghrelin	Cell proliferation	(Ceranowicz et al., 2009; Kasai et al., 2012; Warzecha et al., 2006)
Amhregulin	Surface mucous cell, Zymogenic cells	(Nam et al., 2009)
IFN- γ	Mucous neck cells	(Kang et al., 2005)
Receptors & Others		
EGFR	Mucous neck cells	(Osaki et al., 2010)
Slp 2-a	Surface mucous cells	(Saegusa et al., 2006)
Protease-furin	Surface mucous cells	(Konda et al., 1997)

Surface mucous cells originate from their progenitors at the isthmus and migrate as they mature towards the luminal surface. Their differentiation was probably controlled Trefoil factor family (TFF) 1 peptide. TFFs are mucin associated molecules. TFF1 deficient mice show expansion of surface mucous cells at the expense of parietal cells (Karam et al., 2004). Transforming growth factor α (TGF- α) is a secretory product of surface mucous cells and is involved in their homeostasis (Chen et al., 1993; Coffey et al., 1995; Goldenring et al., 1996; Rutten et al., 1993). Proper differentiation of surface mucous cells depends on the expression of protease furin (Konda et al., 1997) and functional synaptotagmin-like protein-2 (Saegusa et al., 2006). Foxq1 is a transcription factor involved in the biosynthesis of MUC5ac therefore the proper differentiation of surface mucous cells (Verzi et al., 2008).

Parietal cells are the only cells which differentiate at the vicinity of stem cells and their loss affects other cell populations. Parietal cell loss results in expansion of surface mucous cells and depletion of zymogenic cells. GATA-4 and Sonic hedgehog play crucial role in the regulation of cell proliferation and differentiation of parietal cells (Jacobsen et al., 2002, 2005; Waghray et al., 2010). BMP4 has a significant role in the production of parietal cells (Aoki et al., 2011). Gastrin is a trophic hormone which stimulates isthmal cell proliferation and differentiation of both parietal and ECL cells. Hypergastrinemia increases the expression of EGF family members such as heparin binding EGF, amphiregulin, transforming growth factor α in parietal cells and Reg-1 α in chief cells and ECL cells. EGF related peptides inhibit acid secretion and down-regulates parietal cell numbers, but increase surface mucous cell numbers. Inactivating mutations in Reg-1 α , occurs in ECL cell tumors suggesting its role as autocrine growth inhibition although it is a stimulant of the growth of surface mucous cells (Dockray, 1999).

Notch signaling appeared to have a major role in maintaining the tissue homeostasis in the stomach (Kim & Shivdasani, 2011). One of the master regulators of enteroendocrine cells via notch pathway is Hes1 (Jensen et al., 2000). Development of enteroendocrine cells producing gastrin, somatostatin, and glucagon is dependent on neurogenin 3 (Jenny et al., 2002; Lee et al., 2002). In the pyloric antrum, transcription factor ISL-1 is involved in D cell production (Larsson et al., 1995), whereas PDX-1 (Larsson et al., 1996) and Nkx6.1 are involved in G cell production from the G/D common precursor cells. Pax4 and 6 are also known transcription factors in the maturation of antral EC, G and D cells (May & Kaestner, 2010).

Gastric stem cells develop into pre-neck cells and they move downward and gradually proceed into a stepwise differentiation program to form mucous neck cells, pre-zymogenic cells, and finally zymogenic cells (Karam and Leblond, 1993). The molecules involved in the control of these gradual changes leading to the formation of different members of the zymogenic cell lineage are not well documented. In mice, interferon γ was found to induce the secretion of mucus and expression of Muc6, TFF2 and pepsinogenII (Kang et al., 2005). SPDEF is a transcription factor of the ETS family which is initially identified as a regulator of the prostate-specific antigen (Oettgen et al., 2000). In the prostate and breast epithelial cells, SPDEF expression is reported and reduction in its expression is associated with cancer development (Sood et al., 2007). Using the tetracycline inducible over-expression of SPDEF in the intestinal epithelium of adult mice, it has been shown that the SPDEF is sufficient to promote goblet cell differentiation at the expense of other epithelial cell types and to cause profound cell cycle arrest in crypt progenitor cells. In the same study using the colon cancer cell line, the involvement of Notch signaling in

SPDEF induction of goblet cell associated genes is also confirmed (Noah et al., 2010). *In vivo* studies show that in wild type mice SPDEF RNA and protein are expressed in mucous gland cells of the antrum and in mucous neck cells of the glandular corpus (Horst et al., 2010). It is also reported that *in vivo* expression of SPDEF is associated with enhancement in the expression of many genes associated with differentiation and protein glycosylation such as Foxa3, anterior gradient 2 protein (Agr2), glucosaminyl (N-acetyl) transferase 3 in other cell types (Chen et al., 2009).

Agr2 acts as protein disulfide isomerase being involved in controlling ER homeostasis and important for Mucin biosynthesis (Higa et al., 2011; Park et al., 2009). AGR2 expression is also associated with mucous neck cells and inhibition of the differentiation of other lineages from gastric stem cells. Loss of AGR2 expression is associated with mucous neck cell proliferation expressing Sox9 (Gupta et al., 2013).

Mucous neck cell differentiation into zymogenic cells happens through developmentally regulated changes in cell structure directly activating multiple secretory pathway genes that help to establish abundant endoplasmic reticulum and apical accumulation of large secretory granules filled with pepsinogen and other digestive enzymes. The granulogenesis of zymogenic cells requires Mist1 expression (Ramsey et al., 2007). The transcription factor X box binding protein- 1 (XBP1) binds the Mist1 promoter and induces its expression *in vitro* and is also required for the loss of mucous neck cell markers while differentiating into zymogenic cells (Huh et al., 2010). Transgenic expression of Reg protein in mice stomach resulted in enlargement in the proliferative zone and an activity directing the differentiation of parietal and chief cells (Miyaoka et al., 2004). Even though the signaling pathways

of the differentiation programs are not so clear, it is known that many signaling pathways altogether influence the development of stomach where the mesenchymal epithelial interaction is highly essential.

1.6 Stem cells and the origin of cancer

There are two main types of stem cells: embryonic and adult stem cells. Embryonic stem cells gain prime importance due to their pluripotency and differentiation potential to produce all types of body cells (Gattegno-Ho et al., 2012). But their usage has been restricted by many controversies related to their origin and isolation (Keller, 2005). Additional obstacles include safety concerns over potential tumorigenicity and immunocompatibility (Knoepfler, 2009). Adult stem cells are undifferentiated cells residing in many body organs (Barker et al., 2010). A variety of properties enables the study and identification of adult stem cells such as clonogenicity or colony forming unit activity, Hoechst 33342 exclusion property, *in vivo* tissue reconstitution, DNA synthesis, and label retention (Gargett, 2007). Adult stem cells maintain tissue homeostasis by replacing the damaged or dying cells corresponding to the routine cell turnover rates as well as in response to the injured tissues (Li & Xie, 2005). Adult stem cells circumvent many of the ethical and technical issues associated with embryonic stem cells as they can be easily isolated from different tissues and induced to differentiate *in vitro* into multiple cell lineages according to a specific stimulus provided (Singer & Caplan, 2011).

Adult stem cells are not only maintaining homeostasis of the tissue, but they are also capable of repairing it in case of injury (Li & Xie, 2005; Snyder & Loring, 2005). The balance between cell proliferation and differentiation and various signaling molecules control this program to avoid the formation of tumor (Moore &

Lemischka, 2006; Shostak, 2006). It is also reported that in some cases, the mature cells revert back into the proliferative mode for tissue or cell replacement (Dor & Melton, 2004). In this case, the mature cells acquire properties and transcriptional profile of stem cells (Guasch & Fuchs, 2005).

In the stomach, each gastric gland has precise cell composition and turnover rate which is variable in each gastric region. Stem cells in these regions are self-renewing and their differentiation is programed in such a way to meet the need of cell turnover rate and maintain the homeostasis. It is generally believed that alteration in the proliferation rate of gastric stem cells may lead to hyperplastic changes and eventually dysplasia that may progress into cancerous changes. This could happen due to gradual acquisition of genetic or epigenetic mutations in the tumor suppressor genes and oncogenes. Even though the underlying genetics of gastric cancer initiation and progression is not well known, inappropriate activation of Wnt signaling in the pylorus has been reported in subsets of gastric cancer. The conditional ablation of APC tumor suppressor gene initiates proliferation of Lgr5 stem cells leading to adenoma growth in the pyloric region of the mouse stomach (Barker et al., 2010).

Cancer stem cells are either transformed tissue specific stem cells or de-differentiated transit amplifying cells (Sell 2002; Sell & Leffert, 2008). Cancer stem cells are characterized by high levels of cellular efflux pumps and anti-apoptotic proteins, low levels of reactive oxygen species, efficient DNA repair system, and quiescent nature making them resistant to chemo and radiotherapies (Bao et al., 2006; Diehn et al., 2009; Moitra et al., 2011; Todaro et al., 2007). Identification of some molecular markers such as CD133, CD44 (Nosrati et al., 2014) and mutations in E-cadherins also helped in the better understanding and targeting of these cancer

stem cells (Zhao et al., 2015). Strategies applied to eliminate these cancer stem cells includes the antibodies directed against them or inducing their differentiation (Zhao et al., 2015; Zhu et al., 2014).

Several scientists tried to isolate gastric cancer stem cells from patients. Both EpCAM and CD44 surface markers are used for their isolation and transplantation into mice. The xenografts produced heterogeneity in the daughter populations as in the patient's cancer (Chen et al., 2012). CD44 and CD55 are used by other group to isolate cancer stem cells from the patient's blood. CD44 and CD24 are also used for the isolation of gastric cancer cells (Jiang et al., 2012; Zhang et al., 2011).

It seems that gastric cancer can originate from another cellular source. A study in which the gastric epithelium was completely disrupted with lethal irradiation and chronic *Helicobacter pylori* infection, the authors demonstrated that the stomach wall was repopulated with bone marrow-derived stem cells. These mice, in which gastric stem cells were not able to regenerate the disrupted epithelia, eventually developed gastric cancer (Houghton et al., 2004; Guest et al., 2010).

Genetic manipulation in mouse models to alter the proliferation and differentiation program of gastric epithelial progenitors represents a powerful tool and a very useful approach to provide significant clues about the role of gastric stem/progenitor cells in the process of cancer development (Karam, 2010; Karam et al., 2008). The expression of the simian virus 40 large tumor antigen gene under the control of regulatory elements of Atp4b gene specific for the acid-producing parietal cells induced proliferation of the non-cycling pre-parietal cells in developing mice (Karam et al., 1997). When these mice were left to age, the increased proliferation of progenitor cells caused massive hyperplasia with dysplastic changes and eventually the cells became invasive and transdifferentiated into neuroendocrine cells resulting

into neuroendocrine cancer (Syder et al., 2004). In another genetically engineered mouse model, deficiency of trefoil factor (TFF) 1 induces amplification of mucouse cell progenitors which then contributed to the formation of gastric carcinogenesis by eventual invasion into muscularis mucosa (Karam et al., 2004; 2008). These studies highly support the idea of stem cell origin of cancer (Sell, 2002; Sell & Leffert, 2008; Sell et al., 2010).

In humans, examination of the cellular changes that occur during the multistep process of gastric carcinogenesis revealed that alteration of the dynamic program of the proliferating gastric epithelial progenitor cells precedes the development of gastric cancer (Al-Awadhi et al., 2011). This was associated with up-regulation of Oct4 expression in these progenitor cells and alteration in its nuclear translocation in gastric cancer tissues (Al-Marzoqee et al., 2012).

1.7 Gastric cancer

Gastric cancer is very common in many countries (Ferro et al., 2014). It is one of the leading causes of cancer related death worldwide (Parkin, 2001; Parkin et al., 2005). Histologically gastric cancer is classified into two major types: intestinal type and diffuse type. Intestinal type is characterized as clustered, well differentiated and glandular like whereas the diffuse type is infiltrating, poorly differentiated and scattered types (Lauren, 1965). The intestinal type has some correlation with *Helicobacter pylori* infection and is associated with gastritis, intestinal metaplasia and dysplasia. The diffuse type of gastric cancer is thought to develop from the stem cells or progenitors of gastric epithelium (Hohenberger & Gretschel, 2003; Schier & Wright, 2005). Even though the incidence rate of intestinal type of gastric cancer is

declining; the prevalence of diffuse type is reportedly increasing worldwide (Crew & Neugut, 2006).

Gastric cancer is unresectable in more than two-third of its sufferers. The patients with operated gastric cancer have less than 30% chance of 5-year survival and the response rate to chemotherapy in the cases of unresectable tumors is very low (Lordick & Siewert, 2005; Wöhrer et al., 2004).

Surgery is the main therapeutic modality for gastric cancer, although the adverse effects are common. Not only patients diagnosed with gastric cancer may require surgical removal of part or all of their stomach (partial or total gastrectomy), but also some cases of complicated peptic ulcer and abdominal trauma may need gastrectomy. Although gastrectomy has contributed to an improved survival rate for some gastric cancer patients when diagnosed at early stages, the commonly used reconstructions remain inadequate, the quality of life is poor, and morbidity is a major problem in these patients (Bolton & Conway, 2011). The anatomical changes that result after gastrectomy affect the emptying time of the stomach and the digestion of food, leading to a condition known as the postgastrectomy syndrome. These patients usually develop common variable immunodeficiency which causes gastrointestinal problems such as chronic diarrhea, nodular lymphoid hyperplasia and loss of villi leading frequently to malabsorption and malnutrition. Complications of postgastrectomy syndrome include anemia as a result of vitamin B12 or iron malabsorption and osteoporosis (Beyan et al., 2007; Domínguez-López et al., 2011; Williams, 1971). Recent developments in tissue engineering could provide possibilities for improving the quality of life following gastrectomy (Jaklenec et al., 2012).

1.8 Tissue engineering

Tissue engineering is an interdisciplinary field that combines the knowledge and technology of cells, engineering, materials, and suitable biochemical factors to create artificial organs and tissues, or to regenerate damaged tissues (Langer & Vacanti, 1993; Mason & Dunnill, 2008; Orlando et al., 2011). In tissue engineering, cells are taken from a patient and then after expanding their number, seeded onto an appropriate platform to grow *in vitro*. The appropriate stimuli (such as chemical, biological, or mechanical) are applied and over a relatively short time new tissue is formed and implanted to help restore function in the patient. Many reports demonstrated the fabrication and implantation in humans of bioengineered tissue and organs, such as blood vessels (Hibino et al., 2010; L'Heureux et al., 2007; Matsumura et al., 2003; McAllister et al., 2009; Shin'oka et al., 2001; Shin'oka et al., 2005), urinary bladder (Atala et al., 2006), trachea (Baiguera et al., 2010; Macchiarini et al., 2008) and urethra (Raya-Rivera et al., 2011), heart (Ott et al., 2008), liver (Baptista et al., 2009, 2011; Soto-Gutierrez et al., 2011; Uygun et al., 2010), and lung (Ott et al., 2010; Petersen et al., 2010).

Tissue engineering is an emerging topic in biomedical engineering which has shown tremendous promise in creating biological alternatives for harvested tissues, implants, and prostheses. In this approach, the cells are seeded on an artificial extracellular matrix or scaffold and grown to guide their growth and tissues regeneration in three dimensions. The creation of tissues for medical application has already been applied on patients in many institutes. These groundbreaking applications include fabricated skin. The commercial application of a bioartificial skin product for burn treatment was first introduced in 1990 (Miler et al., 1996).

Scaffolds are commonly used in the field of tissue engineering. The scaffold is a platform fabricated from either natural materials, synthetic polymers, or semi synthetic biomaterials (Griffith, 2002). There are protein- and polysaccharide-based natural biomaterials. Collagen, fibrin, and silk are examples for the protein-based natural biomaterials, whereas agarose, alginate, hyaluronan, and chitosan are examples for polysaccharide-based biomaterials. Synthetic-based biomaterials include polymer-based biomaterials, such as polycaprolactone (PCL), polylactic-co-glycolic acid, and polyethylene glycol (Willerth & Sakiyama-Elbert, 2008).

Many studies demonstrated the fabrication of scaffolds with different structure and topography varying from spongy nature to gel or to form a complex hybrid structures involving pores, channels and embedded peptide sequences. The new material processing strategies allow the production of a variety of scaffolds, such as porous, non-porous and fibrous scaffolds. When the cells are grown on 2D platform, they can proliferate, but their differentiation potential would be limited (Knight & Przyborski, 2014). Therefore, porous or fibrous 3D scaffolds showed a great potential for tissue engineering and clinical applications.

There are several requirements in the design of scaffolds for tissue engineering. In addition to being biocompatible both in bulk and degraded form, these scaffolds should possess appropriate mechanical properties to provide the correct stress environment for the new tissues. Also, the scaffolds should be porous and permeable to permit the ingress of cells and nutrients, and should exhibit the appropriate surface structure and chemistry for cell attachment (Freed et al., 2006; Pham et al., 2006). The scaffold should not be toxic to cells and biodegradable with balanced degradation rate and non-toxic metabolites as the end products. It should allow cell attachment and migration and have the capacity to deliver and retain the

cells and biochemical factors. The scaffold provides a framework and initial support for the cells to attach, proliferate and differentiate and form an extracellular matrix (Agrawal & Ray, 2001; Sachlos & Czernuszka, 2003). The porosity of the scaffold is an essential factor. Adequate porosity allows the diffusion of vital nutrients, promotes vascularization, and when transplanted, encourages angiogenesis (Ratner et al., 2004). The high porosity of the scaffold will allow cell migration and good cell adhesion (Kim & Mooney, 1998; Salgado et al., 2004). Finally, the engineered scaffold should not elicit an immune response while remaining a viable framework for cellular infiltration/proliferation, and contributing the complex function of the native extracellular matrix (Matthews et al., 2002; Sell et al., 2008).

1.9 PCL scaffolds

PCL is an aliphatic polyester and the ring-opening polymerization of *ε*-caprolactone yields a semi crystalline polymer with a melting point of 58–63°C and a glass transition temperature of 260°C (Woodruff & Hutmacher, 2010). The repeating molecular structure of PCL homopolymer consists of five nonpolar methylene groups and a single relatively polar ester group. This structure gives PCL unique properties that are similar to polyolefin because of its high olefinic content, while the presence of hydrolytically unstable aliphatic-ester linkage causes the polymer to be biodegradable (Yang et al., 2011). This polymer has been regarded as tissue compatible and frequently used as a biodegradable suture.

PCL is a biodegradable and biocompatible polymer and is widely used in biomedical applications as a drug delivery carrier or scaffold for a variety of cell types. Importantly, PCL has been approved by the Food and Drug Administration (Ekaputra et al., 2011; Porter et al., 2009). PCL degrades by hydrolytic scission with

resistance to rapid hydrolysis via its hydrolytic aliphatic-ester linkage and lose is average of 50% for different treatments of its strength in 4 weeks using an *in vitro* degradation test (Johnson et al., 2009). Degradation times can extend for up to 24 months. PCL scaffolds alone, without co-blending of other polymers, yield mechanical properties adequate for craniofacial bone repair. Additionally, PCL scaffolds support mesenchymal stem cell attachment, proliferation, osteogenic differentiation, and aid in bone repair of critical sized rabbit cranial defects (Endres et al., 2003; Schantz et al., 2003; Zhou et al., 2007). The degradation, mechanical strength, and biocompatibility properties make PCL an excellent polymer for long-term tissue engineering (Cheung et al., 2007). PCL is one of these biodegradable polymers that have been extensively studied for various biomedical applications (Kweon et al., 2003; Williams et al., 2005; Woodruff & Hutmacher, 2010). The PCL polymer was found to be very promising for growth of different types of stem cell in both soft and hard tissues (Dai et al., 2004; Shor et al., 2007; Yeong et al., 2010).

1.10 Mouse gastric stem (mGS) cell line

The mGS cell line is established less than a decade ago (Farook et al., 2008) from a transgenic mouse expressing SV40 large T antigen using the promoter of H,K-ATPase gene (Li et al., 1995). These mice were characterized by an amplified population of gastric epithelial progenitor cells since early stages of their development (Karam et al., 1997). From one of these mice, the stomach was dissected and the gastric epithelial cells were harvested using a simple collagenase/EDTA method. When these cells were plated in RPMI culture medium, some attached and started to grow in small groups and eventually formed a monolayer. Then they were trypsinized and re-cultured several times. Finally, a

clone of these cells was isolated and maintained in culture for more than 100 passages (Farook et al., 2008).

The mGS cells were stained positive for an epithelial cytokeratin. Electron microscopy revealed that these cells have junctional complexes like epithelial cells. Also, they showed high nucleus to cytoplasmic ratio, many free ribosomes, short microvilli and few small cytoplasmic organelles such as rough endoplasmic reticulum, Golgi apparatus and mitochondria (Farook et al., 2008). All these features are similar to those of stem cell population previously described in mouse stomach (Karam and Leblond, 1992). On the other hand, these cells did not bind to any of the differentiation markers known for mature gastric epithelial cells: antibodies specific for intrinsic factor, chromogranin A, H,K-ATPase α and β -subunit, and lectins specific for surface mucous and gland mucous cells (*Griffonia simplicifolia* or GSII and *Ulex europaeus* agglutinin or UEA, respectively). In support of the progenitor/stem cell nature of these cells, they were found to express Notch3, DCLK1, and Oct4 (Giannakis et al., 2008; Al-Marzooq et al., 2012). With the availability of such a cell line which represents the gastric epithelial stem cells, it becomes possible to explore their use as an *in vitro* model system for gastric epithelial tissue engineering.

1.11 Gastric tissue engineering

Although numerous gastric replacement techniques with different enteric reservoirs have been applied to improve the quality of life of patients after total gastrectomy, the optimal reconstruction remains controversial (Speer et al., 2011). Recent advances in the field of tissue engineering allowed fabrication of many tissues and organs. As an alternative remedy to the post-gastrectomy issues, tissue engineered stomach that replaces the mechanical and metabolic functions of a normal

stomach have been proposed. If this technological progress is achieved, it would benefit many patients undergoing gastrectomy.

Directed differentiation of embryonic pluripotent stem cells into a variety of cell types opens a promising avenue for cell replacement therapy and provides a powerful tool for basic translational research (Green et al., 2010). With the restrictions on the use of human embryonic stem cells in Japan, scientists were successful in reprogramming of adult somatic differentiated cells to form induced pluripotent stem (iPS) cells and, therefore, paved the way for the technology of generating patient-specific pluripotent cells (Yamanaka et al., 2009).

Little is known about the engineering of stomach tissue. The few studies available in the literature employed a very similar strategy for the regeneration and repair of stomach in animal models. In one study, organoid units, described as mesenchymal cores surrounded by epithelia, were isolated from rats and transplanted para-topically on biodegradable polymer tubes, and eventually implanted intraperitoneally into syngeneic hosts. The tubes were pre-coated with collagen type I. Four weeks later, engineered stomachs were found to have a well-developed gastric epithelium including gastric pits and express α -actin smooth muscle and gastrin (Grikscheit et al., 2003).

In another study, a short segment of the stomach was resected from a 6-week-old swine (Sala et al., 2009). Organoid units (defined as multicellular clusters with predominantly epithelial content) were isolated and loaded onto biodegradable scaffold tubes as described in the previous study (Grikscheit et al., 2003). The constructs were then implanted intraperitoneally in the autologous host. Seven weeks later, implants were harvested and found to be similar to the antrum of a native

stomach with alcian blue-positive mucous cells and expressing smooth muscle actin in the muscularis mucosa (Sala et al., 2009).

Maemura et al (2003), also used isolated organoid units from rat stomach and them on biodegradable polymer tube made up of polyglycolic acid coated with poly-L-lactic acid. The implanted construct formed neomucosa and smooth muscle layers as demonstrated by immunohistochemistry using anti-mucin and -proton pump antibodies. The same group in 2004 transplanted the polyglycolic acid microporous tubes seeded with the gastric epithelial organoid units isolated from the columnar epithelial area of stomach of 7-day-old neonatal Lewis rats to adult Lewis rat. The surface topology of stomach resembled that of a native stomach (Maemura et al., 2004). Maemura et al in (2008) studied the potential of tissue engineered stomach to function as a food reservoir following total gastrectomy. In this study, they have used the rat model in which the neonatal stomach organoids seeded polyglycolic acid based microporous tubular scaffold coated with polylactic acid is transplanted in the omental area of the abdominal cavity. After three weeks of transplantation, the normal stomach was resected out and the cephalic side of the newly developed stomach is cut open as a hole and anastomosed to the native esophagus while the caudal end is opened longitudinally in order to remove its contents and anastomosed to the distal site of native jejunum. After 24 weeks, the secretory function of the tissue-engineered stomach was confirmed using immunohistochemical staining (Maemura et al., 2008).

In 2011, Speer and coworkers used isolated mouse gastric organoids and demonstrated by immunohistochemistry a highly differentiated stomach cells containing mucous, endocrine, chief, and parietal cells. Tissue-engineered stomach

epithelium also demonstrates proliferation and the expression of two putative gastric stem cell markers: DCAMKL-1 and Lgr5 (Speer et al., 2011).

In brief, it seems that studies available in the literature used gastric organoids made of mesenchymal (connective tissue) cells including blood vessels and the gastric epithelial cells. So, with the availability of mGS cell line, it will be interesting to generate a synthetic scaffold to establish a 3D culture model that could be useful for gastric tissue engineering and also to dissect the molecular events involved in the differentiation of gastric stem cells into mature cells.

1.12 Aim of the project

The overall goal of this research project was to produce new knowledge regarding the adult stem cells of the stomach which, throughout the life of organism, are responsible for generating different cell lineages secreting mucus, pepsinogen, hydrochloric acid, and various hormones. In humans and rodents, these stem cells are few in number and difficult to isolate or investigate. Even though some evidences suggest that they play an important role in the development of gastric cancer, little is known about these stem cells. The factors involved in their early commitment program into different cell lineages are not known. It is not also known whether they have potential for use in gastric tissue engineering.

Specific Objectives:

- i)** To generate and characterize various forms of PCL scaffolds,
- ii)** To characterize the growth and viability of mGS cells on these scaffolds,
- iii)** To assay for proliferation and differentiation of mGS cells on the most suitable form of PCL scaffolds for possible use in gastric epithelial tissue engineering,
- iv)** To investigate the effect of acidic pH on the growth and differentiation of mGS cells grown on 2D and 3D culture conditions
- v)** To define some molecular factors involved in the commitment and differentiation program of mGS cells grown on 3D culture condition.

Chapter 2: Materials and Methods

2.1 Preparation of PCL Scaffolds

Synthetic PCL with a molecular number (M_n) of 70,000-90,000 by GPC (Sigma-Aldrich, USA) was used in this study as the starting material for scaffold preparation. Initially, a homogeneous solution containing 25% PCL (by weight) in chloroform was used as a stock solution for the preparation of three different forms of scaffolds (Fig.3).

Nonporous PCL scaffolds were prepared by casting 10 mL of the stock solution into a flat Petri dish, then left in the air for complete dryness. Microporous PCL scaffolds were prepared by casting 10 mL PCL solution containing 50 % (w/v) NaCl (with an average size of ≤ 50 microns), as a porogen, in a flat Petri dish, then air-dried to remove any remaining solvent. Each PCL sheet was soaked in de-ionized water with stirring to leach out NaCl granules leaving behind a microporous scaffold. Microfibrous PCL scaffolds were prepared by electrospinning technique (Fig.4). Details of the electrospinning process are mentioned previously (Laurencin et al., 2006; Bhattacharyya et al., 2006). Briefly, a 10 mL of 25% PCL solution was spun at an applied voltage of 12 kV, a spinning distance of 14 cm, and a feeding rate of 0.16 mL/min. Electrospun PCL scaffolds were kept in air to ensure complete dryness.

2.2 Scanning electron microscopic (SEM) analysis of scaffolds

Dry scaffolds were processed for gold palladium coating. Morphologies of the scaffolds were evaluated using SEM (XL-30 Phillips, Amsterdam, Netherlands) at an accelerating voltage of 15 kV. The scaffolds were examined at different

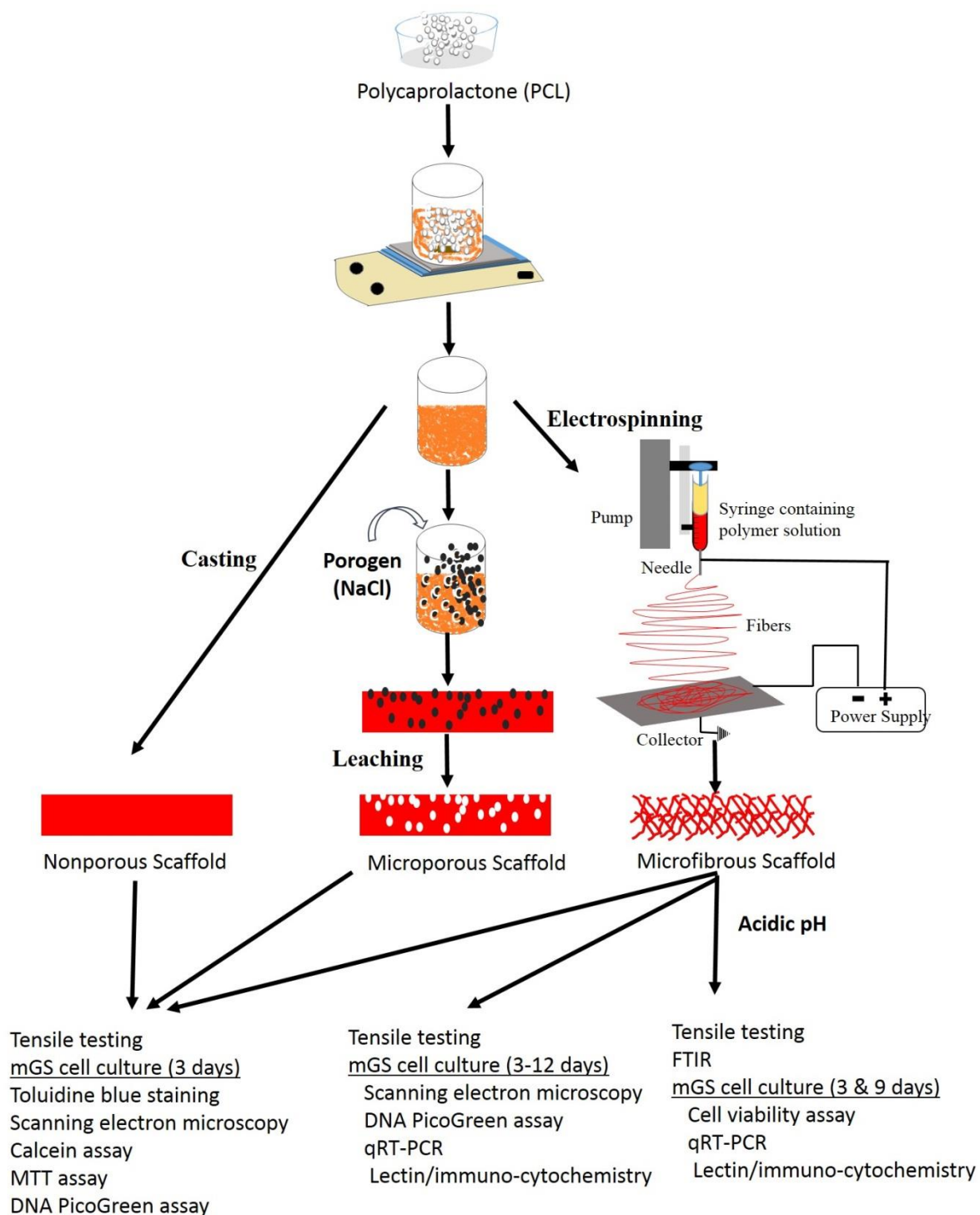


Figure 3: Summary diagram of the preparation of 3 different types of PCL scaffolds (nonporous, microporous, and microfibrous) and their use in mGS cell culture for different time points and assays.

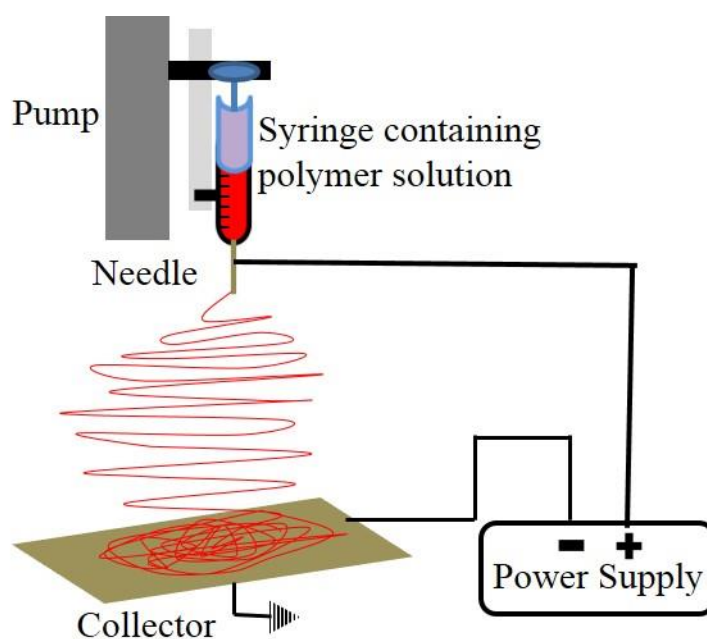


Figure 4: Diagram representing the process of electrospinning. A syringe is filled with the polymer solution and connected to a pump. The needle is connected to anode. The solution comes out of the needle as fibers which are collected onto the metallic plate connected to cathode. The fibers are deposited on the plate randomly generating a sheet of fibrous polymer.

magnifications and electron micrographs were taken for each type of scaffolds. The topographical features of the nonporous, microporous, and microfibrous scaffolds including pore size, pore distribution, fiber size and distribution were studied and compared using SEM micrographs.

2.3 Measurement of the tensile strength of the scaffolds using universal mechanical testing machine (MTS)

Mechanical tests were carried out to evaluate the tensile behavior and mechanical integrity of prepared nonporous, microporous and microfibrous PCL scaffolds. The tests were conducted using universal testing machine MTS with a load cell of 100 kN under displacement controlled conditions. All tests were conducted under overhead speed of 5 mm/min and at room temperature. Caliper measurements were used to determine scaffold thickness. Scaffolds were cut into rectangular strips of 5 x 2 cm. Tensile strength measurements were carried out in triplicate according to published procedure (Mourad, 2010). For comparison, 6-month-old C57BL/6 mouse stomach tissues (n = 3) were collected, washed in cold phosphate buffered saline (PBS), and immediately tested for their tensile strength. SEM examination was also conducted on the scaffolds before and after the tensile tests to investigate the effect of applied load and deformation on the morphology of the scaffolds.

2.4 Experiment 1: Culture of mGS cells on different PCL scaffolds for 3 days

A frozen aliquot of mGS cells was thawed and seeded in a tissue culture flask containing 10% serum in RPMI media. Cells were allowed to grow till semi-confluent in a 37°C incubator adjusted to 5% CO₂ and 95% O₂. The culture media was changed every other day. Cells were passaged twice to stabilize their

morphology and growth rate. The mGS cells were then seeded (1.6×10^5 cells) on each sterilized nonporous, microporous and microfibrinous PCL scaffolds (5 mm in diameter) placed inside 96 well plate. After 3 days of culture, the cells were processed in triplicate for different procedure

2.4.1 Toluidine blue staining for light microscopy

The mGS cells were fixed in 4% paraformaldehyde for 15 min, washed with PBS, then incubated in 1% toluidine blue solution for 30 sec. Cells on the different scaffolds were then washed in double-distilled water and examined with inverted microscope (Olympus, Tokyo, Japan).

2.4.2 SEM analysis

To examine surface morphology of mGS cells grown on different PCL scaffolds, cells were fixed in 4% paraformaldehyde for 15 min, washed in PBS and post-fixed in 1% osmium tetroxide for 10 min. Following dehydration in ascending grades of ethanol, cells were processed for gold-palladium coating, and finally examined with Phillips SEM.

2.4.3 Cell viability (Calcein assay)

The mGS cells were incubated for 30 min with 2 μ M calcein in PBS at 37°C. The absorbance of calcein was detected at 485-535 nm using VICTOR™ X3 PerkinElmer 2030 multilabel plate reader. For statistical analysis, the one way ANOVA with Dunnet Multiple Comparison Test model was employed. Graphical representation of the data (mean \pm SD) was performed using GraphPad Prism software (La Jolla, CA, USA).

2.4.4 Metabolic activity (MTT assay)

The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells. Nonporous, microporous and microfibrous PCL scaffold were cut into 0.5 cm² size and sterilized. 1.6×10^6 mGS cells were seeded and cultured for 3 days in 10% FBS containing RPMI media on 96-well plate. Then, 10 μ l (5 mg/ml) of 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 3-4 hr at 37°C in the dark. After the incubation, 100 μ l of dimethyl sulfoxide (DMSO) was added to each well to break down the formazan crystals. The absorbance was measured at 560 nm. The readings obtained were plotted on a graph using GraphPad software and the values were analyzed using one way ANOVA, Tukey multiple comparison test. P values less than 0.05 were considered to be significant.

2.4.5 Cell quantification using DNA PicoGreen assay

The mGS cells were suspended in serum-containing RPMI and seeded (5×10^5 cells) onto pre-sterilized nonporous, microporous and microfibrous PCL scaffolds (15 mm diameter) placed in a 24-well tissue culture dish and allowed to grow for 3 days in a 37°C incubator adjusted to 5% CO₂ and 95% O₂. After 3 days, the cultured media were collected and spun down at 10,000 rpm for 3 min and the pellet stored at -80°C in 1 ml of Milli-Q water. The DNA was extracted from the samples by repeated freeze-thaw cycles followed by ultrasonication using Sonic Ruptor 250 Ultrasonic Homogenizer (Omni International, Kennesaw, GA, USA). For

quantification of DNA, Quant-iT PicoGreen dsDNA kit (Invitrogen, Eugene, OR, USA) was used according to the manufacturer's instructions. Briefly, a five-point standard curve of 1000, 100, 10, 1 and 0 ng/ml Lambda DNA was prepared. Following 5 min incubation of sonicated samples with the PicoGreen dye at room temperature, the intensity of fluorescence was measured at 520 nm on the PerkinElmer reader. For statistical analysis, the one way ANOVA with Tukey Multiple Comparison Test model was employed. Graphical representation of the data was performed using GraphPad software.

2.5 Experiment 2: Culture of mGS cells on microfibrous PCL scaffolds for 3, 6, 9, and 12 days

The mGS cells were suspended in serum-containing RPMI and seeded (2.5×10^5 cells) onto pre-sterilized microfibrous PCL scaffolds (15 mm diameter and 0.9 mm thickness) placed in a 12-well tissue culture dish and allowed to grow in a 37°C incubator containing 5% CO₂ and 95% O₂. The culture medium was changed every other day. After 3, 6, 9 and 12 days, cultured cells were processed for quantification of DNA and gene expression analysis.

2.5.1 Cellular quantitation using DNA PicoGreen assay

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. For quantification of DNA, the Quant-iT PicoGreen dsDNA kit was used according to the manufacturer's instructions and as mentioned above. The intensity of fluorescence was measured at 520 nm using the PerkinElmer reader. Scaffolds without cells were used as blank samples. For statistical analysis, a one

way ANOVA with Tukey Multiple Comparison Test model was employed. Graphical representation of the data was performed using GraphPad Prism.

2.5.2 Gene expression analysis using quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The mGS cells were seeded on pre-sterilized scaffolds (1.5 cm diameter) placed in 24-well plate with 10% RPMI media. After 3, 6, 9 and 12 days of culture, RNA was isolated either from cells on scaffolds using RNeasy kit according to manufacturer instruction (Qiagen, Hilden, Germany). The final RNA was treated with DNAase and quantified using a ND-1000 spectrophotometer (NanoDrop, Wilmington, USA). The cDNA first strand synthesis was carried using GoScript reverse transcription kit (Promega, Madison, WI, USA) and Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The qRT-PCR was carried out using the SYBR Green method and the QuantStudio 7 Flex real-time PCR system (Applied biosystems) using primers listed in table 2. The expression levels were determined in triplicate and normalized using glyceraldehyde 3-phosphate dehydrogenase (Gapdh).

2.5.2.1 RNA Extraction

The scaffolds with cells cultured for each time point were washed in cold PBS. Then, 600 µl of RNA lysis buffer was added. The lysates were collected into 1.5 ml microcentrifuge tubes. Equal volume of 70% ethanol was added to each lysate. The mixture was transferred into a spin column and centrifuged at 10,000 rpm for 15 sec. Spin column membrane was washed at the speed of 10,000 rpm for 15sec.

RNA was eluted using 30µl of nuclease free water at 10,000 rpm for 1min and quantified using NanoDrop spectrophotometer. Isolated RNA were stored at -80°C.

2.5.2.2 First Strand cDNA Synthesis

The RNA (2µg) was added to random primers (0.5µg/reaction) and the volume was made up to 10 µl with nuclease free water in 0.2 ml PCR tube and heated at 70°C for 5min. The tubes were immediately chilled on ice after the reaction. Then, 10 µl of the reverse transcription reaction mix was added to each tube. The reaction was carried out for annealing at 25°C for 5 min and extension at 42°C for 1 hr followed by the inactivation of reverse transcriptase enzyme at 70°C for 15 min in thermal cycler. Samples of the synthesized cDNA were stored at -20°C.

2.5.2.3 qRT-PCR

Real-time PCR for the cDNA samples were performed using the SYBR Green method and the primers listed in the table 2. Non-template controls were run in parallel. The reaction was carried out for activation of AmpErase UNG activation at 50°C for 2min, activation of Ampli TaqGold DNA polymerase at 95°C for 2 min and denaturation at 95°C for 15 sec followed by the annealing and extension at 60°C for 1min. All results were normalized against the house keeping gene GAPDH. Gene expression were analysed using $\Delta\Delta C_T$ method and the fold difference were calculated using $2^{-\Delta\Delta C_T}$.

Table 2: List of gene-specific primers used for quantitative RT-PCR studies

Gene	Forward primer	Reverse primer
GAPDH	TCAAGAAGGTGGTGAAGCAGG	TATTATGGGGGTCTGGGATGG
DCAMKL1 (DCLK1)	CAGCCTGGACGAGCTGGTGG	TGACCAGTTGGGGTTCACAT
OCT4	TAGGTGAGCCGTCTTTCCAC	GCTTAGCCAGGTTTCGAGGAT
PCNA	CGTCTCACGTCTCCTTGGTACAG	GGACATGCTGGTGAGGTTACAC
HK-ATPase- α	TGTACACATGAGGTCCCCTTG	GAGTCTTCTCGTTTTCCACACC
MUC5ac	AGGGCCCAGTGAGCATCTCCTA	CATCATCGCAGCGCAGAGTCA
GASTRIN	GGACCAGGGACCAATGAGG	CCAAAGTCCATCCATCCGTAGG
SPDEF	GTTGCCTGCTACTGTTCCCAGATG	AAAGCCACTTCTGCACGTTACCAG
XBP-1	GAAAGCGCTGCGGAGGAAAC	GAGGGGATCTCTAAAACTAGAGGC
RAB3d	AGTGTGACCTGGAAGACGAAC	CCAGGGATTTCATTCATCTTGT
MIST-1	TGGTGGCTAAAGCTACGTGTC	GACTGGGGTCTGTCAGGTGT

2.6 Experiment 3: Culture of mGS cells on microfibrinous PCL scaffolds for 3 and 9 days

The mGS cells were suspended in serum-containing RPMI and seeded on microfibrinous PCL scaffolds placed in a 12- or 24-well tissue culture plate similar to that described in experiment 2. Cells were analysed after 3 and 9 days culture as follows:

2.6.1 SEM analysis

To examine surface morphology of mGS cells grown on microfibrinous PCL scaffolds for 3 and 9 days, they were fixed in paraformaldehyde and processed for SEM as mentioned in experiment 1.

2.6.2 Multi-label immuno- and lectin-cytochemical analysis

The cells grown on scaffolds for 3 and 9 days were fixed in 4% paraformaldehyde for 15 min. Following three PBS washes, cells attached to scaffolds were incubated in 20% buffered sucrose overnight at 4°C. The cell-containing scaffolds were then mounted on an aluminum stalk using Shandon cryomatrix (Thermo Fisher Scientific, Waltham, MA, USA) and orientated perpendicular to the plane of sectioning. Samples were then dipped in liquid nitrogen for a few seconds. Using a cryostome FSE cryostat (Thermo Scientific, Cheshire, UK), 10-30 micron-thick sections were obtained and mounted on gelatin-coated slides. Some cryosections were stained with hematoxylin and eosin and adjacent sections were probed with various biomarkers.

Some cryosections were first processed for haematoxylin and eosin staining for orientation and general morphology. Cryosections were kept at room temperature for 30 min and washed in distilled water. The sections were stained with hematoxylin for 2 min and extra stain was washed out with tap water and then treated with acid alcohol and washed again with distilled water for 10 min. Tissue sections were stained with eosin for 30 sec and washed by dipping in distilled water followed by dehydration in a series of ethanol, 70%, 90%, 95% (15 sec each) and 100% for 2 min with 2 changes and clearing in xylene. Finally, the sections were mounted using DPX and coverslip to examine under the microscope.

Cryosections obtained from mGS cell growing on scaffolds for 3 and 9 days were processed for lectin binding and immuno-cytochemistry. Following incubation with blocking solution (1% bovine serum albumin in PBS) for 60 min, cells were incubated overnight with the following mono- or polyclonal antibodies specific for: H,K-ATPase alpha and beta subunits (for parietal cells, mouse monoclonal, Medical & Biological Laboratories, Nagoya, Japan), TFF1 (for surface mucous or pit cells), TFF2 (for mucous neck or gland mucous cells), chromogranin (for enteroendocrine cells, mouse monoclonal, DAKO, Glostrup, Denmark), ghrelin (for a subgroup of enteroendocrine cells). Anti-TFF1, -TFF2 and -ghrelin mouse monoclonal antibodies are gifts from Dr Catherine Tomasetto, Strasbourg, France. The dilutions used for all antibodies were 1:50 or 100. Probed sections were washed in PBS and the appropriate biotinylated anti-mouse or anti-rabbit immunoglobulin G was added as a secondary antibody for the primary antibodies mentioned above. Finally, Alexa Fluor (555 or 488)-conjugated avidin was added to visualize the antigen-antibody binding sites using inverted fluorescence Olympus microscope or Nikon Eclipse 80i confocal microscope (Tokyo, Japan). Cryosections of the cells were also incubated

for 60 min with fluorophore-conjugated *Ulex europaeus* agglutinin (UEA) I lectin (specific for surface mucous cells), *Griffonia simplicifolia* (GS) II lectin (for mucous neck cells), or *Dolichos biflorus* agglutinin (DBA) (Falk et al. 1994; Karam et al. 2005). All lectins were purchased from Sigma (St. Louis, MO, USA) and used at dilution of 1:100.

2.7 Experiment 4: Culture of mGS cells in acidic pH using 2D and 3D systems

Since the future plan of this project is to use the mGS cells growing on PCL scaffolds for *in vivo* animal experiments to test their possible use for regenerative therapy, it is necessary to examine first how these cells will grow in acidic environment comparable to that of the stomach and whether or not the acidic pH will affect the PCL scaffold.

2.7.1 Effect of acidic pH on the viability of mGS cells in 2D culture

A frozen aliquot of our immortalized mGS cells was gradually thawed and seeded in a tissue culture flask containing 10% serum in RPMI media. Cells were passaged a couple of times to stabilize their morphology and growth rate. Cells were then trypsinized, washed in PBS, re-suspended in serum-containing RPMI, and seeded onto 96-well tissue culture plate (2000 cells per well), and allowed to grow in an incubator adjusted to 5% CO₂ and 95% O₂. After reaching 60% confluence, the culture media was replaced with same media, but at different pH: 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5. The pH values of the media were monitored and adjusted by using 1.0 N HCl. After 5-hr incubation in presence of 5% CO₂ and 95% O₂, the cells were processed for calcein viability assay using live/dead cell staining kit (Molecular Probes, Grand Island, NY, USA) as mentioned before. The cells were

incubated for 30 min with 2 μ M calcein and propidium iodide at 37°C. The absorbance of calcein and propidium iodide were then detected at 485-535 nm and 530-620 nm, respectively using PerkinElmer reader. For statistical analysis, the one way ANOVA with Dunnet Multiple Comparison Test model was employed. Graphical representation of the data was performed using GraphPad Prism software. Both dead and viable cells were also examined using the Olympus fluorescence microscope.

2.7.2 Effect of acidic pH on mGS cell migration in 2D culture

The mGS cells were seeded on 6-well plates and after reaching semi-confluence, a scratch was made in each plate with a tip of 1ml sterile pipette. After PBS wash, the cells were incubated with 10% RPMI media for 1hr. Then the plates were treated with 10% RPMI media of pH 6.0. In the control plate, wounded cell layer was growing in pH 7.4. Cells migrating to close the wound were photographed using 10X objective lens of Olympus inverted microscope in all wells and the width of the wound was measured after 1 hr and 1, 2 and 3 days.

2.7.3 Effect of acidic pH on microfibrous PCL scaffolds

To test whether the acidic environment has any effect on the mechanical properties and chemical composition of the scaffolds, several scaffold samples were incubated for 3, 6, 9 and 12 days in RPMI media at different pH values: 3.0, 5.5 and 7.4. Some scaffold samples were left dry and used as control. Control and media exposed samples were all processed for both mechanical and chemical testings.

2.7.3.1 Mechanical testing using MTS

The scaffolds were tested for their mechanical properties namely tensile strength, stress, and strain by using the universal testing machine MTS with a load of 5 kN under displacement controlled conditions. All testes were carried out under overhead speed of 5 mm/min and at room temperature.

SEM examination was also conducted on the samples (as previously mentioned) before and after tensile tests to investigate the effects of acidic pH on the morphology and orientation of the microfibrinous scaffolds after tensile testing.

2.7.3.2 Chemical testing using fourier transform infrared (FTIR) spectroscopy

Scaffolds with 0.5 cm diameter incubated in 500 μ l RPMI media at pH 3.0, 5.5 and 7.4 for 3-12 days were collected after each time point. Scaffolds were immediately washed in Milli Q water, dried overnight, and analyzed using FTIR spectrometry (Shimadzu, Kyoto, Japan) to test whether the acidic environment has any deleterious or degradation effects on the PCL material.

2.7.4 Effect of acidic pH on mGS cells cultured on microfibrinous scaffold

2.7.4.1 Cell Viability of mGS cells cultured on scaffold at acidic pH

Three sets of microfibrinous scaffold were cut into 0.5 cm size and placed in 96 well plates. Scaffolds were sterilised in 70% ethanol for 1hr followed by 1hr UV air dry. The scaffolds were washed in PBS for 30 min and incubated overnight in media. 1.6×10^5 cells were seeded per scaffold and allowed to grow for 24hr in RPMI media containing 10% FBS at pH 7.4. On the next day the 10%FBS containing RPMI media was changed with RPMI media at pH 3.0 and 5.5. For control set, media at pH 7.4 was used. The cells were allowed to grow in tissue culture incubator for 5 hr

and the viability was checked by incubating the scaffold with 2 μ M calcein and propidium iodide for 30 min and the fluorescence intensity was measured. The graph and statistical analysis were prepared using Graph Pad Prism software. Microscopic images showing live and dead cells were also taken using the fluorescence microscope.

2.7.4.2 Quantitative RT-PCR of mGS cells in 2D culture at acidic pH

The mGS cells were seeded on tissue culture plate. After 24 hr exposure to normal 10% RPMI media of pH 7.4, the media were replaced with 10% RPMI media of pH 5.5 and incubated for 3 and 9 days. After 3 and 9 day of culture, RNA was isolated using RNeasy kit and quantified as mentioned before. qRT-PCR was carried as mentioned before using primers listed before.

2.7.4.3 Quantitative RT-PCR of mGS cells in 3D culture at acidic pH

The mGS cells were seeded on pre-sterilised 1.5 cm diameter scaffold placed in 24-well culture plate. After 24 hr exposure to normal 10% RPMI media of pH 7.4, the media were replaced with 10% RPMI media of pH 5.5 and incubated for 3 and 9 days. Then RNA was isolated from cells on scaffolds using RNeasy kit. The final RNA was treated with DNAase and quantified. qRT-PCR was carried out as mentioned before.

2.7.4.4 Immuno- and lectin-cytochemistry of mGS cells cultured on microfibrous scaffolds at acidic pH

The mGS cells grown on scaffolds for 3 and 9 days incubated with 10% RPMI media of pH 7.4 and 5.5 were fixed in 4% paraformaldehyde for 15 min. Following three PBS washes, cells attached to scaffolds were processed for cryosectioning as mentioned before. Some cryosections were stained with hematoxylin and eosin and adjacent sections were probed with various biomarkers. To test whether cellular phenotype was affected by acidic pH, lectin binding and immune cytochemistry were performed on cryosections as mentioned before using lineage-specific antibodies: anti-H,K-ATPase, -TFF1, -TFF2, -chromogranin antibodies. As a control, mGS cells grown on coverslips or chamber slides and mouse stomach tissue sections were probed with similar lectins and antibodies.

Chapter 3: Results

In this study, three different forms of PCL scaffolds were prepared using different methods. These scaffolds were characterized and tested for growth of mGS cells. To evaluate the suitability of mGS cells cultured on PCL scaffolds for possible *in vivo* and/or clinical applications, the effects of an acidic environment on both cells and scaffolds were analyzed.

3.1 Characterization of PCL Scaffolds

3.1.1 Morphological Features

SEM examination of the three different types of scaffolds revealed a significantly different surface topography. The nonporous scaffolds were characterized by patterned irregularities probably due to evaporation of the solvent during air-drying (Fig. 5a). In contrast, the microporous scaffolds prepared using NaCl as porogen appeared to have many homogeneously distributed pores which had variable sizes (50 to 100 nm) and frequently appeared interconnected (Fig. 5b). The sheets of microfibrous scaffolds prepared by the electrospinning technique were approximately 0.9 mm in thickness. They appeared as a complex meshwork of microfibers which were variable in diameter, 8-20 microns (Fig. 5c). Moreover, high magnification SEM micrographs clearly revealed the rough surface and porosity of the microfibers (Fig. 5d).

3.1.2 Mechanical Features

Mechanical tests were carried out to evaluate the tensile behavior and mechanical integrity of PCL scaffolds (nonporous, microporous and microfibrous).

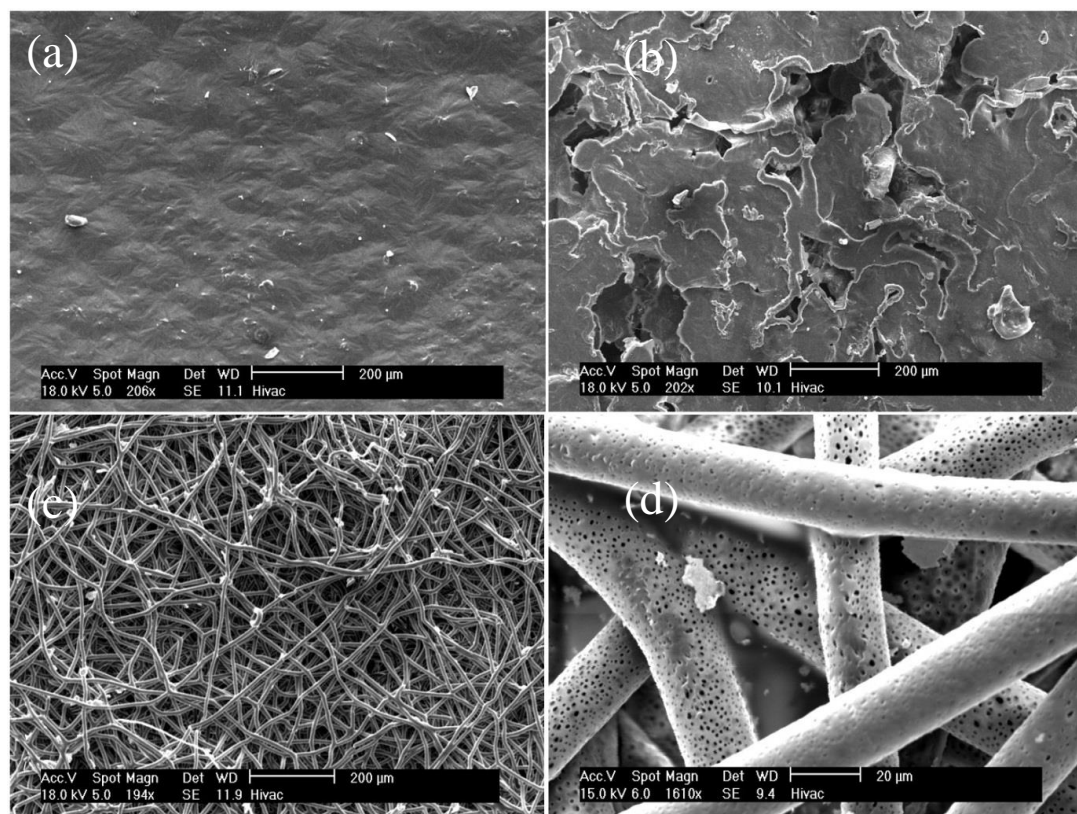


Figure 5: SEM micrographs of nonporous (a), microporous (b) and microfibrinous (c,d) scaffolds showing their surface topography. Note the moderate roughness of the nonporous scaffold (a). The microporous scaffold appeared to have numerous pores variable in size and frequently appeared interconnected (b). The microfibrinous scaffold appeared like a complex meshwork of microfibrs which were variable in thickness (c) and reveals some surface roughness (d). Bar = 200 μ m (a,b,c), 20 μ m (d).

Figure 6 shows images of nonporous scaffold and microporous scaffold samples before (Figs. 6a, c, 7c) and after (Figs. 6b,d, 7d) conducting tensile tests respectively. Each sample had a total length of 50 mm, gage length of 25 mm, and width of 4 mm. The thickness of the samples varied from 0.75 mm for nonporous, and 1.0-1.7 mm for microporous scaffolds. Microfibrous tensile test samples had the same length and gage dimensions and were 0.9 mm in thickness. All samples have been fractured in the gage length except in the case of microfibrous scaffolds. To compare the PCL scaffolds with animal tissue, the mechanical integrity (stress and strain) of the mouse stomach was also tested. The mouse stomach was cut open and clamped in between the handles of the machine. Figure 7 shows images of stomach wall samples before (Fig 7a) and after (Fig 7b) tensile testing.

To visualize the effect of the tensile testing on the topographical appearance of the microfibers of PCL scaffolds, small samples were processed before and after testing for SEM examination. Figure 8 shows the SEM micrographs of microfibrous scaffolds before (Fig. 8a) and after (Fig. 8b) the tensile testing. The random orientation of the microfibers was clearly evident before testing (Fig. 8a), whereas after conducting the tensile test, the microfibers became oriented in the direction of loading (Fig. 8b). It was also clear that the fibers were still maintaining their integrity at accepted level of interconnections. This characteristic mechanical property of the microfibrous scaffolds depicts that they are flexible and can sustain the effects of deformation and load.

The stress-strain curves obtained for the 3 types of scaffolds revealed different patterns. The tests were conducted using the same universal material testing system (MTS) with a load cell of 5 kN under displacement controlled conditions. All tests were conducted under overhead speed of 1 mm/min and at room

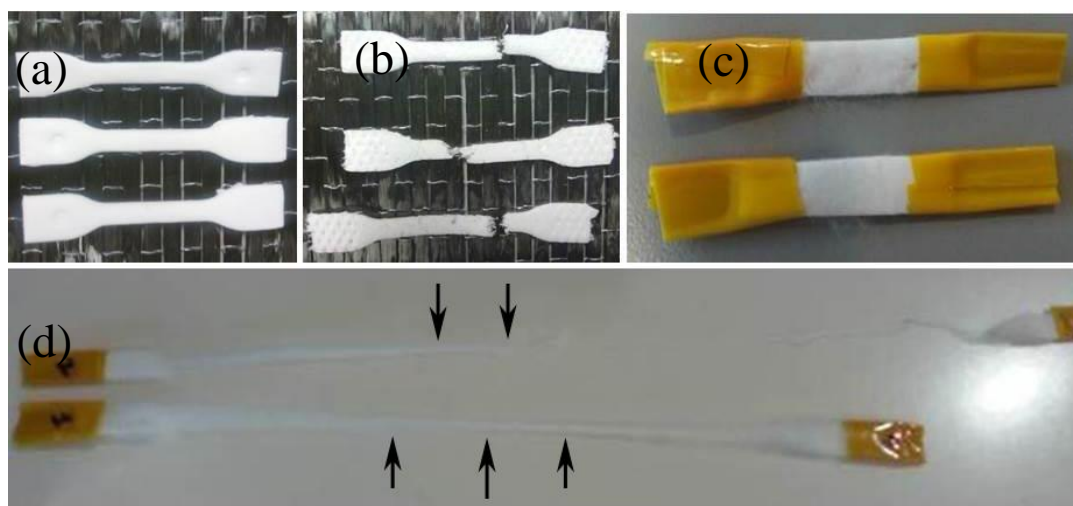


Figure 6: Representative samples of nonporous (a, b) and microfibrinous (c, d) PCL scaffolds before (a, c) and after (b, d) tensile testing.

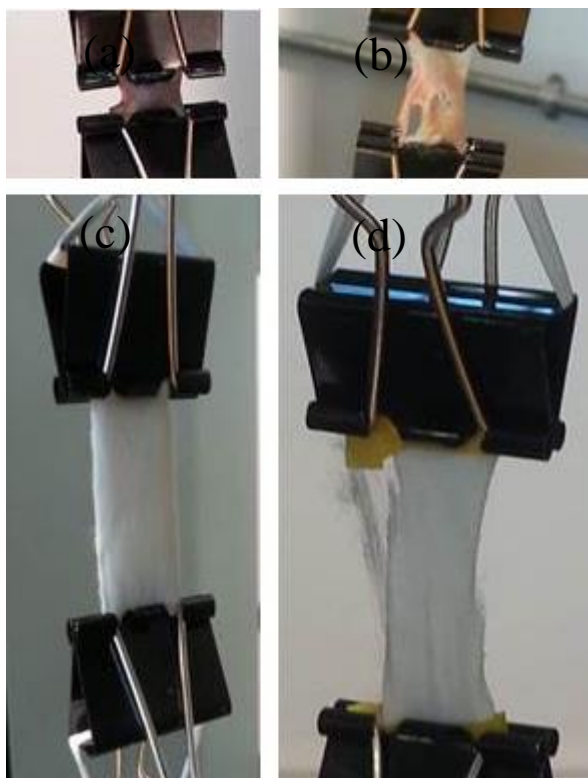


Figure 7: The mouse stomach wall (a, b) and microfibrous PCL scaffold (c, d) samples as they appear before (a, c) and after (b) or during (d) tensile testing. Note the stretch and lacerations that appeared in the stomach wall at the end of performing the mechanical testing. The scaffold at the end of the test appeared like in Fig. 7d

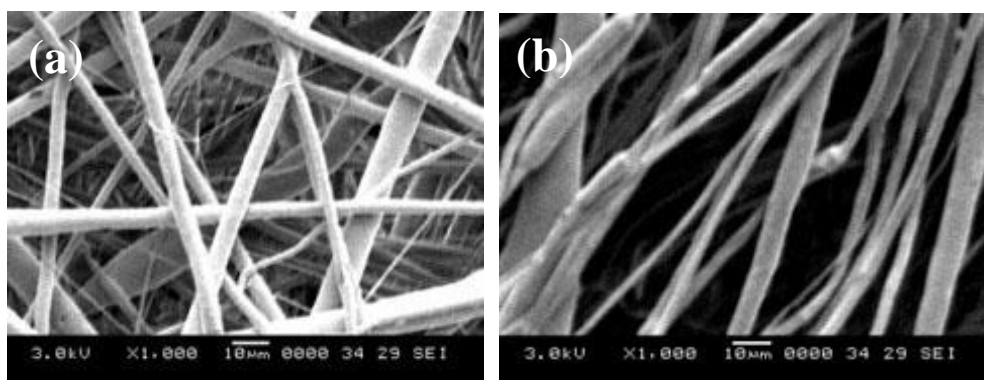


Figure 8: SEM images of microfibrinous PCL scaffold samples before (a) and after (b) conducting the tensile test. Note the random arrangement of microfibrils before testing (a) and the elongated fibers oriented in one direction after the testing (b).

temperature. Figure 9a demonstrates typical tensile test curves of four nonporous samples. The maximum achieved stress load was in the range of 5 up to 8 MPa and the percent strain ranged from 40 to 75. This reflects good toughness (strength and deformation) of the nonporous PCL scaffolds.

The tensile curve of microporous scaffold (Fig. 9b) showed the maximum stress of 2.5 - 3.5 MPa with a percent deformation ranging from 25 to 47. The porosity of the scaffold played a role in the change in load bearing capacity which was expected. Therefore, in comparison to nonporous scaffold, microporous scaffold showed better flexibility.

The tensile performance of microfibrous scaffolds (Fig. 9c) showed the maximum stress of 0.35 to 0.65 MPa and percent deformation of 1200-1400. These samples showed more flexibility and fewer loads than nonporous and microporous scaffolds. The stress-strain values of microfibrous scaffold indicated the best mechanical flexibility and the ability to sustain a wide range of load and deformation among the samples tested. In contrast, the mouse stomach tissue (Fig. 9d) showed maximum stress of 0.18 MPa with a percent deformation of 110%. Despite the relatively low stress durability, these values reflected the flexibility of the stomach wall and the little load it can bear.

For further comparison of the 3 types of scaffolds and the stomach wall, the peak stress (tensile strength) and peak strain of the stomach and scaffold samples were estimated (Table 3). The mouse stomach tissue showed a lower peak stress than all types of PCL scaffolds. The closest peak stress to that of the stomach wall was the PCL microfibrous scaffold which showed a 3-fold higher peak stress and 1.1 fold higher peak strain compared to that of the stomach wall. In contrast, nonporous and microporous scaffolds showed much higher peak stresses (41.4 and 18.6,

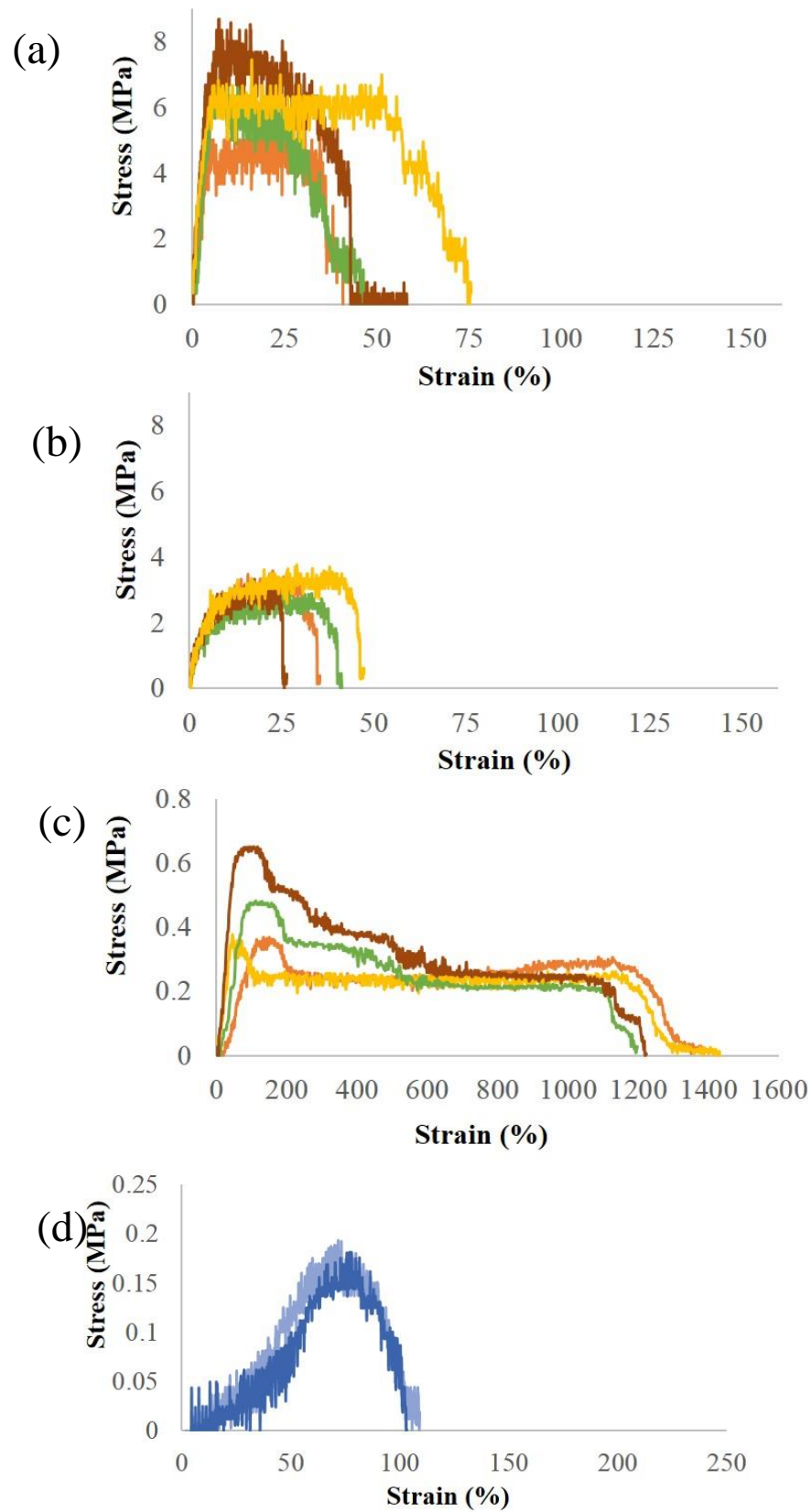


Figure 9: Stress-strain curves of nonporous (a), microporous (b), and microfibrinous (c) PCL scaffold samples and also for the mouse stomach wall (d).

respectively) and much lower peak strain and lower flexibility under tensile testing compared to microfibrinous scaffolds. Therefore, the higher flexibility of microfibrinous scaffolds makes them closer to natural gastric tissues than nonporous and microporous scaffolds. The proximity of the microfibrinous scaffolds in terms of mechanical properties to the wall of the stomach makes them well suited for further studies.

3.2 Characterization of mGS cells cultured on PCL scaffolds for 3 days

(Experiment 1)

Because the mGS cells were cultured and passaged many times since they were first established and studied, it was necessary to first test whether they would bind to any of the lectins and antibodies known to be specific for differentiated mouse gastric epithelial cells. Therefore, mGS cells grown on coverslips to 50% of confluence were fixed with 4% paraformaldehyde, permeabilized, and then probed with lectins and antibodies. While mGS cells did not bind to GSII, UEA, and DBA lectins (Figs. 10b-d), they reacted with WGA lectin (Fig. 10a). Binding with WGA was cytoplasmic and intensified in the perinuclear and Golgi area. When mGS cells were probed with antibodies specific for trefoil factor peptides (TFF1 and TFF2), chromogranin, ghrelin, H,K-ATPase, and intrinsic factor, they did not show any immunoreactivity (not shown).

3.2.1 Light microscopic features

Microscopic examination of the toluidine blue-stained mGS cells revealed their variable appearance on the different types of scaffold used (Figs. 11a-c). On day 3, the cells grown on nonporous and microporous scaffolds appeared at low density

Table 3: Tensile performance (stress and strain) of different PCL scaffolds as compared to mouse stomach tissue.

The data are presented as mean \pm SD

Samples	Peak stress (MPa)	Peak strain (%)
Nonporous scaffold	6.50 \pm 1.20	13.7 \pm 2.5
Microporous scaffold	2.93 \pm 0.36	28.5 \pm 5.0
Microfibrous scaffold	0.49 \pm 0.12	162.5 \pm 14.4
Mouse stomach tissue	0.15 \pm 0.01	147.5 \pm 9.5

with small colonies (Figs. 11a, b). However, on the microfibrinous scaffolds the cells tended to appear at high density (Fig. 11c).

3.2.2 SEM features

SEM analysis was also used to characterize the morphological appearance of mGS cells and to describe their shape and size. On the nonporous and microporous scaffolds, the cells were few, small, and stellate in shape with a convex surface (Figs. 12a,b). When mGS cells were grown on microfibrinous scaffolds they were also small but most of them appeared flattened (Fig. 12c). These flattened cells had cytoplasmic processes spanning the space between microfibers, and therefore, attached to more than one microfiber. Some cells appeared to be attached to only one microfiber.

3.2.3 Cellular viability and quantification

When mGS cells were seeded on nonporous, microporous and microfibrinous PCL scaffolds and maintained for 3 days, the pattern of cell growth varied on the different scaffolds. The viable growing mGS cells were assayed by using the calcein live-cell labeling method. Measurement of the intensity of fluorescence produced by the viable cells attached to the scaffolds showed a moderate labelling for the cells growing on nonporous or microporous scaffolds. However, the cells growing on microfibrinous scaffolds showed very high labelling (Fig. 13). Therefore, it seems that microfibrinous scaffold supported growth of mGS cells more than nonporous and microporous scaffolds. Statistical analysis of the data confirmed that cell labeling was significantly higher ($p < 0.0001$) on microfibrinous than nonporous or microporous

scaffolds (Fig. 13). This finding clearly demonstrated the suitability of microfibrous scaffold for mGS cell growth.

In order to account for both cells attached to the scaffold and those suspended in the media, another cell viability method was applied using MTT. The mGS cells were analysed following their 3-day growth on different types of scaffold. The MTT reagent was added to the RPMI media and then the colorimetric reading for living cells attached to the scaffold as well as suspended in the media were obtained. The highest colorimetric reading was produced by the cells growing on microfibrous scaffolds and, therefore, confirming the preferential growth of mGS cells on microfibrous scaffold (Fig. 14).

Since the unattached cells suspended in the media could be either live or dead cells, it was necessary to quantify their total number. This was carried out by DNA isolation and quantification. Following 3-day culture of mGS cells on nonporous, microporous, and microfibrous scaffolds, the RPMI media were collected and spun down to separate floating cells. The pelleted cells were processed for DNA quantification using the PicoGreen assay. Measurements showed more amount of DNA on nonporous and microporous scaffolds when compared to microfibrous scaffolds. Statistical analysis of the data showed that cell attachment was significantly higher ($p < 0.0015$) on microfibrous (**) than nonporous or microporous scaffolds (Fig.15). The difference between the amount of DNA in cells attached to nonporous and microporous were not statistically significant ($p > 0.05$).

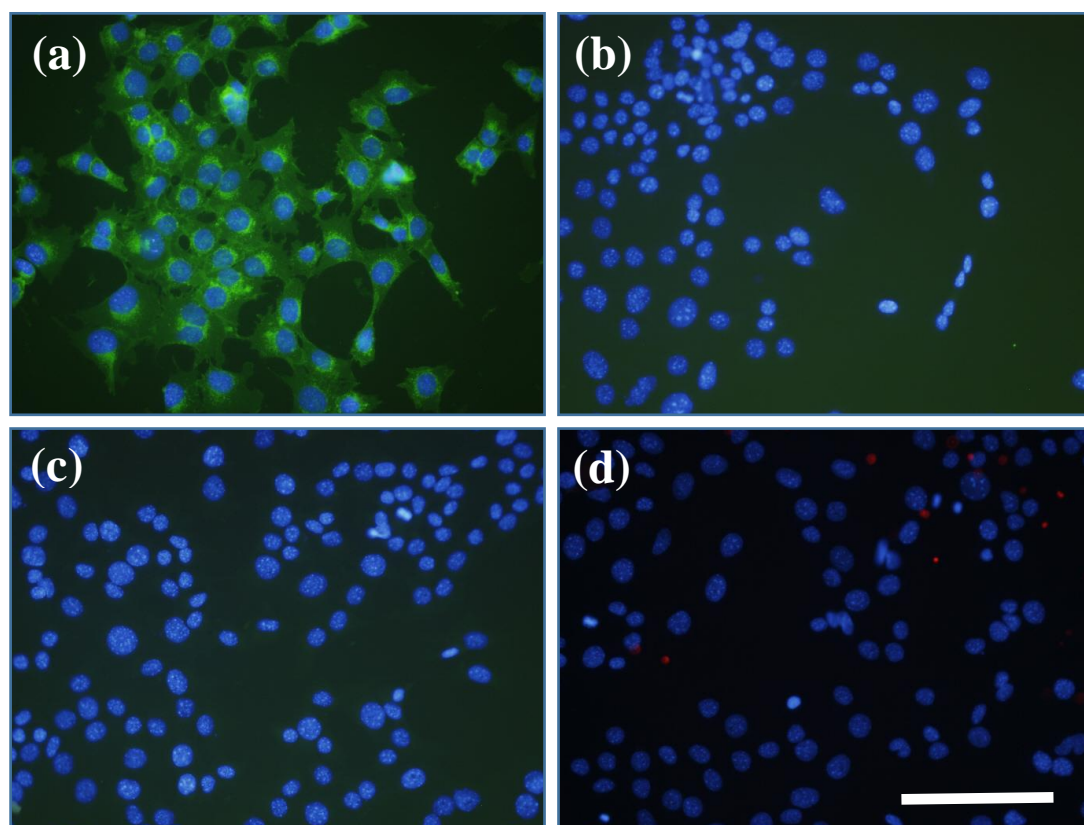


Figure 10: Lectin cytochemistry for the mGS cells cultured on coverslips. Fluorescence micrographs show the blue nuclear staining with DAPI (a, b, c, d) and the binding of WGA (green) (a). The cells are stained negative for GSII (b), UEA (c), and DBA (d). Scale bar = 50 μ m (a-d)

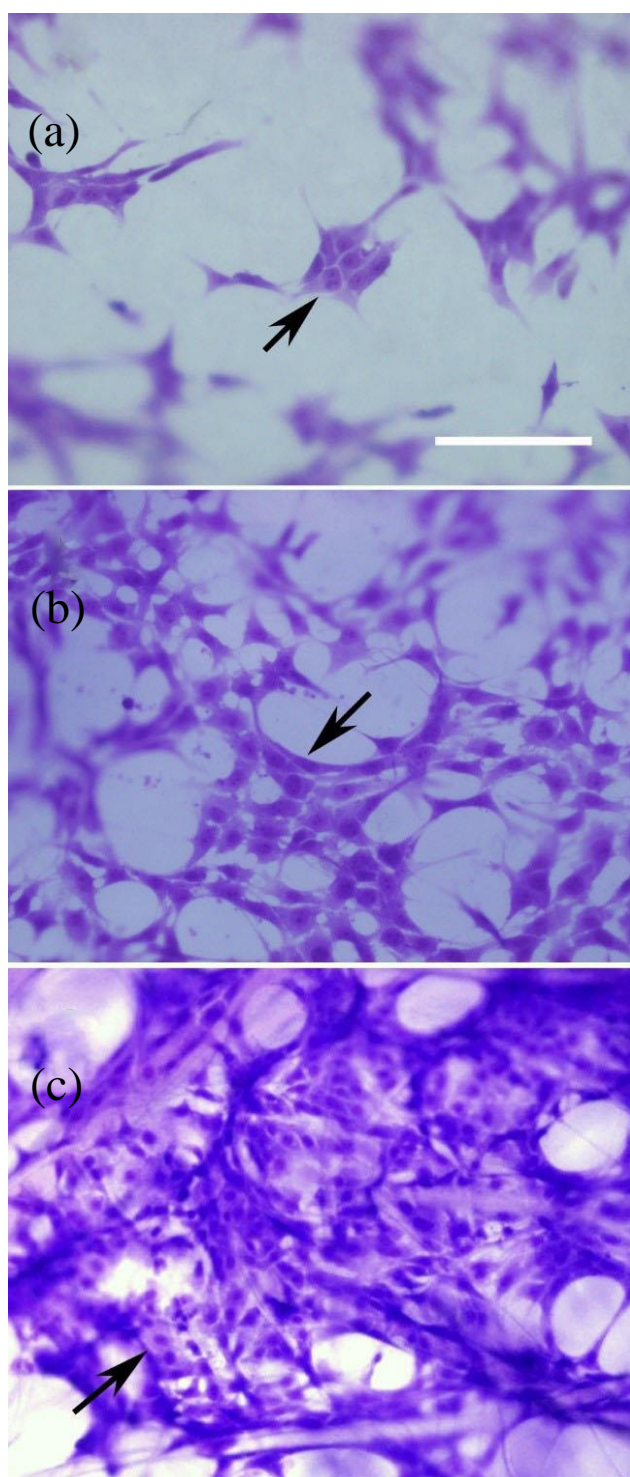


Figure 11: Light micrographs of toluidine blue-stained mGS cells after 3 days culture on the surfaces of nonporous (a), microporous (b), and microfibrous (c) PCL scaffolds. Arrows are pointing to groups of cells stained with toluidine blue. Bar = 50 μm (a–c).

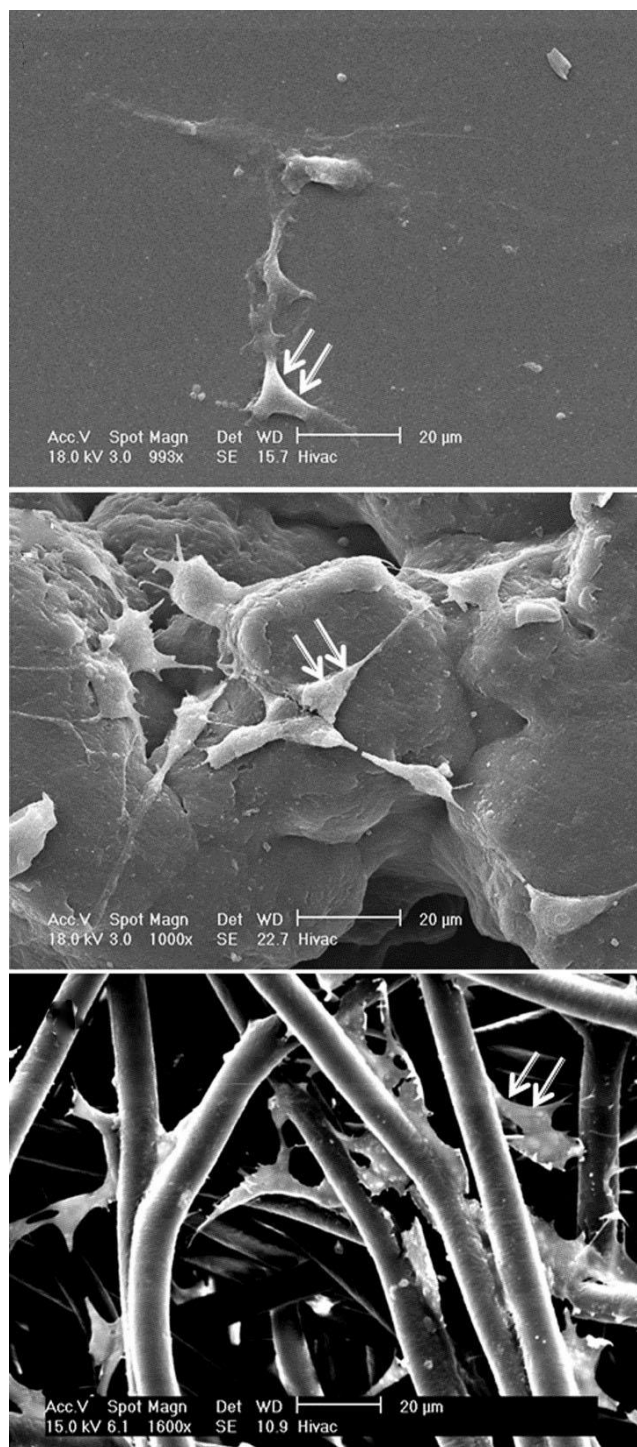


Figure 12: Scanning electron micrographs of mGS cells cultured on nonporous (a), microporous (b), and microfibrous (c) PCL scaffolds for 3 days. Note that mGS cells (arrows) are attached to each other and to the surfaces of the scaffolds or microfibers. Bar = 20 µm (a–c).

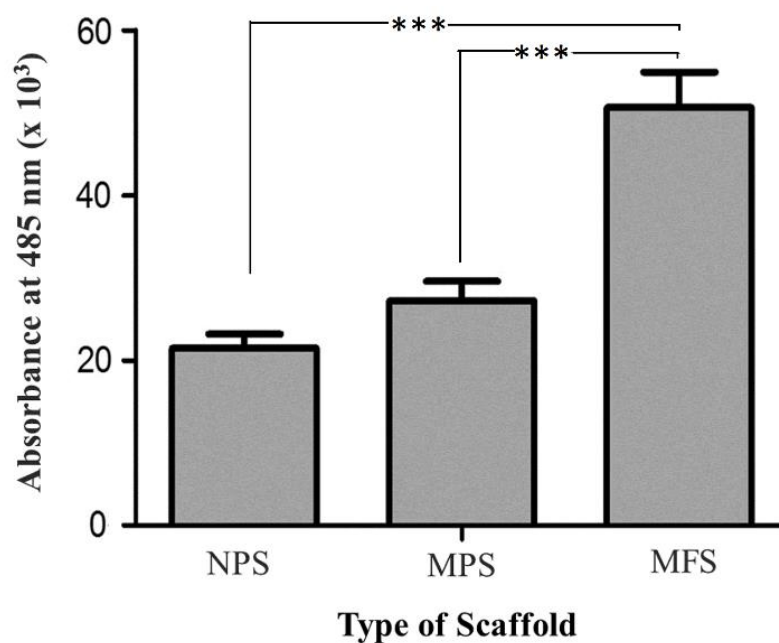


Figure 13: Cell viability assay for mGS cells after 3 days of culture on different types of scaffolds: nonporous (NPS), microporous (MPS) and microfibrous (MFS). Note absorbance values representing cell viability are low in case of cells growing on NPS and MPS, but significantly increase in case of MFS. Data expressed as mean \pm SD. *** $P < 0.0001$.

3.3 Characterization of mGS cells cultured on microfibrinous PCL scaffolds for different time points (Experiment 2)

Since mGS cells preferentially grew on microfibrinous scaffolds, it was interesting to follow the seeded cells after 3, 6, 9, and 12 days and determine the pattern of their growth. The attached cells at different time points were lysed and their DNA was extracted and quantified using PicoGreen assay. These data would reflect the number of cells attached and grown on the scaffolds at different days of culture. As shown in figure 16, the measurements revealed that the amount of DNA increased from 539 ng/ml (day 3) to 720 ng/ml (day 6), indicating the growth or increase in number of the attached mGS cells from day 3 to day 6. However, when the cells were cultured for 9 days, the amount of DNA (reflecting the number of cells) was significantly reduced ($p < 0.05$) as shown in figure 16. A reduction in the amount of DNA was also observed in cells cultured for 12 days with insignificant change in the amount of DNA which indicated no significant change in the number of cells (Fig. 16).

3.4 Characterization of mGS cells grown on microfibrinous PCL scaffolds for 3 and 9 days (Experiment 3)

The increase in the amount of DNA extracted from mGS cells grown on microfibrinous scaffolds up to 6 days and its decrease on day 9 could suggest either some cell death and/or inhibition of cell proliferation, induction of cell differentiation, and detachment of differentiated cells. Therefore, it was necessary to analyze mGS cells at day 9 and compare them with those of day 3.

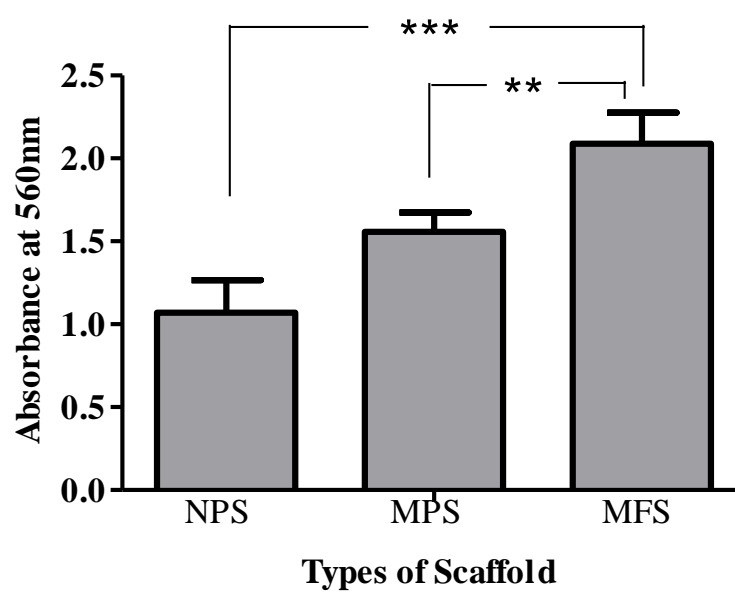


Figure 14: Cell metabolic activity assay using MTT reagent for mGS cells after 3 days of culture on different types of polycaprolactone scaffolds: nonporous (NPS), microporous (MPS) and microfibrous (MFS). Data expressed as mean \pm SD. *** $P < 0.0001$; ** $P < 0.001$.

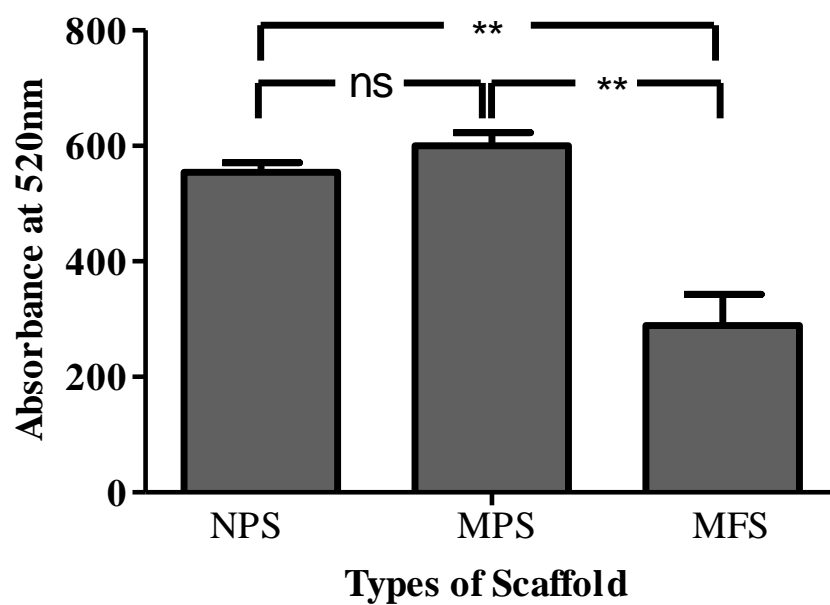


Figure 15: DNA PicoGreen assay for quantification of unattached mGS cells after 3 days of culture on 3 types of scaffolds: nonporous (NPS), microporous (MPS) and microfibrous (MFS). Data expressed as mean \pm SD. ** $P < 0.0015$.

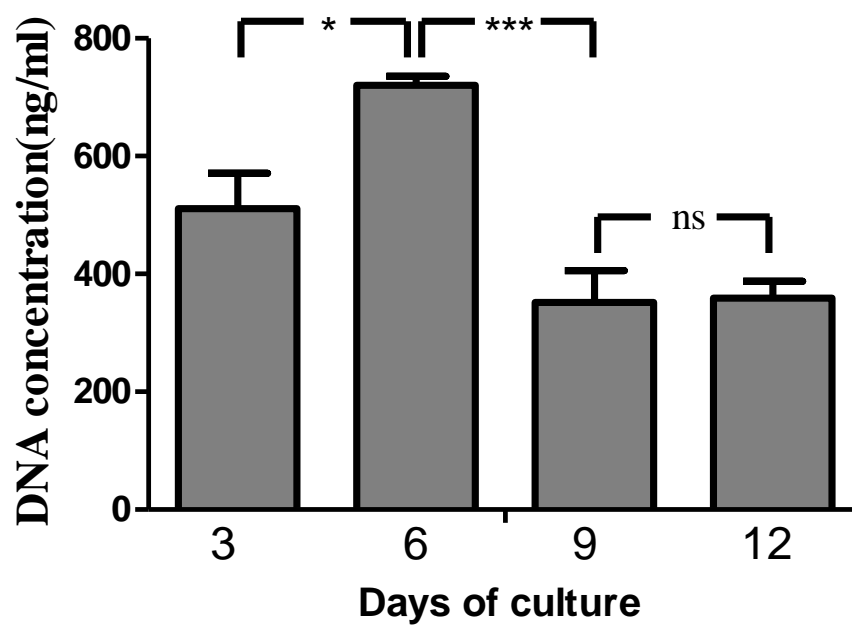


Figure 16: Estimation of DNA content of mouse gastric stem cells cultured on microfibrous polycaprolactone scaffolds for 3, 6, 9 and 12 days using PicoGreen assay. Data expressed as mean \pm SD. *** $P < 0.0001$.

3.4.1 Morphological features

Scanning electron microscopic examination of the mGS cells revealed their stellate or polyhedral shape and small size on day 3 (Fig. 17a). Their cytoplasm appeared flattened. By day 9, mGS cells attached to the microfibers of the scaffold appeared to be expanded or enlarged in size (Fig. 17b). The cytoplasm of mGS cells also appeared flat, but extended between the microfibers of the scaffold.

3.4.2 Gene expression analysis using qRT-PCR

RNA was extracted from mGS cells grown on tissue culture plate and from mGS cells grown on microfibrinous PCL scaffolds for 3, 6, 9 and 12 days. The purified RNA was processed for reverse transcription assay and cDNA was utilized for gene expression analysis using specific primers and qRT-PCR. The expression level of a specific gene was determined in triplicate for each sample and normalized to the expression of GAPDH which did not significantly differ in the various samples.

The growth of mGS cells on microfibrinous scaffold was associated with a gradual down-regulation in the mRNA level of genes specific for pluripotency, Notch signaling, and proliferation of stem cells. The level of Oct 4 expression in mGS cells indicated that they maintained their stemness and pluripotency at any day (3-12) of culture (Fig. 18a). However, the level of Oct4 was maximum at day 3 of culture and was reduced thereafter suggesting a decline in the stemness or pluripotency of mGS cells.

The expression level of DCLK1 mRNA was gradually up-regulated in mGS cells cultured for 3 to 12 days on microfibrinous scaffold (Fig. 18b). The expression of PCNA gene was down-regulated indicating a reduction in the capacity of mGS cells

to proliferate when cultured on the microfibrinous scaffolds (Fig. 18c). This change in the proliferation program of mGS cells could be an indication of cell differentiation.

In addition to genes specific for stem cells and cell proliferation, it was also important to study the expression of some transcriptional factors involved in the differentiation of the mucous neck and zymogenic cell lineage, such as SPDEF, Rab3d, XBP1 and Mist. Interestingly, the level of SPDEF mRNA expression showed a gradual stepwise up-regulation with the days of culture and became significant by days 9 and 12 ($p < 0.001$, $p < 0.0001$, respectively) (Fig. 19a). In addition, the XBP1 expression was significantly down-regulated (Fig. 19b). The transcripts of Mist1 and Rab3d were not detected in any of the samples at any time point.

3.4.3 Lectin- and immuno-cytochemical analysis

To test whether the reduction of cell number and the associated increase in cell size were due to cell differentiation, cryostat sections of mGS cells grown on microfibrinous scaffolds for 3 and 9 days were processed for lineage-specific lectin binding and antibody probing using histo- and immuno-cytochemistry.

Expressions of glycoconjugates and proteins that bind to lineage-specific lectins and antibodies, respectively, were taken as a measurement of cell differentiation. Microfibrinous scaffolds with mGS cells cultured for 3 and 9 days were sectioned at 10-30 μm thickness and mounted on gelatin-coated slides. Some sections were stained with hematoxylin and eosin for light microscopy and general morphology (Fig. 20a). Adjacent sections were processed for immunoprobng using anti-TFF2 antibodies specific for gland mucous cells. The results revealed that after 9 days of mGS cell culture on microfibrinous PCL scaffolds, some cells expressed TFF2 (Fig. 20b). Adjacent sections were also probed with fluorophore-conjugated

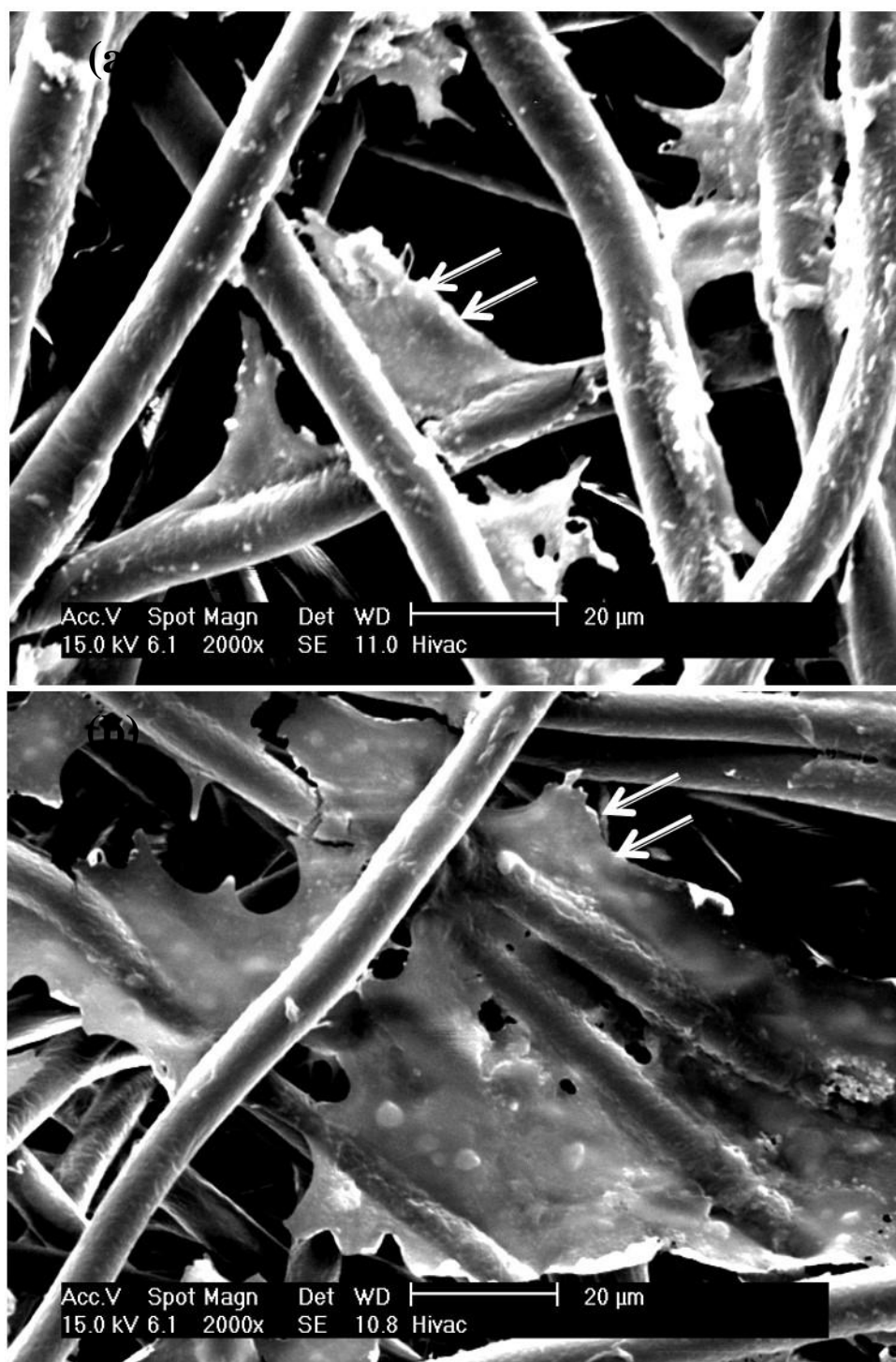


Figure 17: Scanning electron micrograph of mouse gastric stem cells cultured on microfibrillar polycaprolactone scaffolds for 3 (a) and 9 (b) days. Cells appear polyhedral or stellate after 3 days (arrows) and adhere to the microfibrils and after 9 days expand and fill many of the spaces between microfibrils. Bar = 20 µm (a, b)

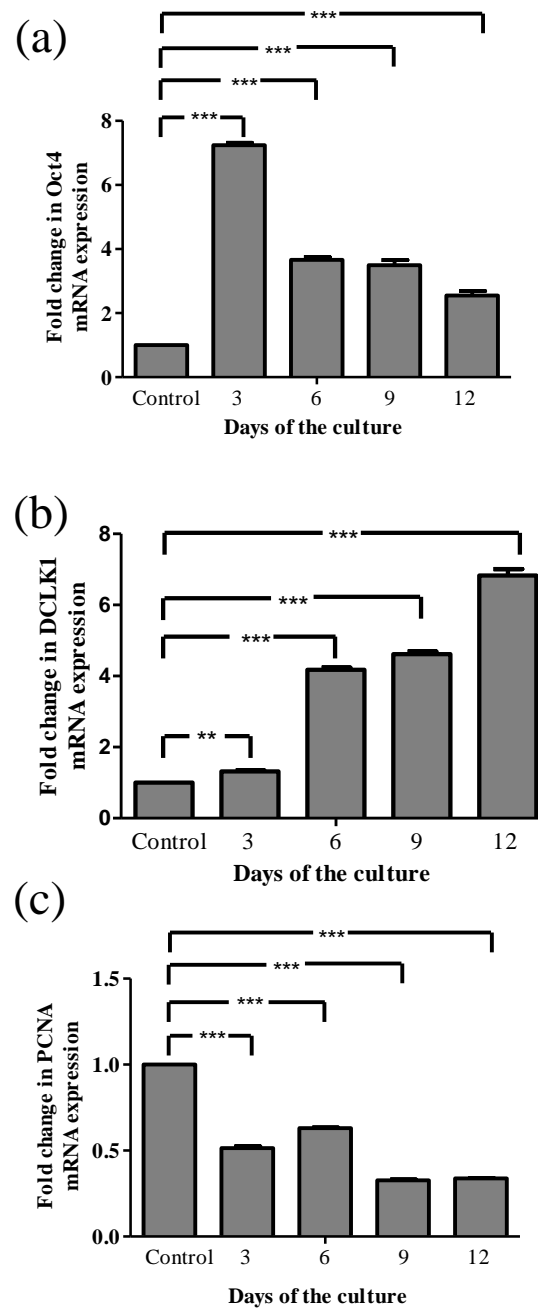


Figure 18: mRNA expression of Oct4 (a), DCLK1 (b), and PCNA (c) in mGS cells grown on culture plate (control) and on microfibrous scaffolds for 3, 6, 9, and 12 days and normalised with GAPDH. Oct4 expression is up-regulated in cells growing on scaffolds; by about 7-fold at 3 days (a). DCLK1 is up-regulated with days of culture in a step-wise pattern reaching 7-fold increase by day 12 (b). PCNA expression is significantly down-regulated (b). **= $p < 0.001$; ***= $p < 0.0001$.

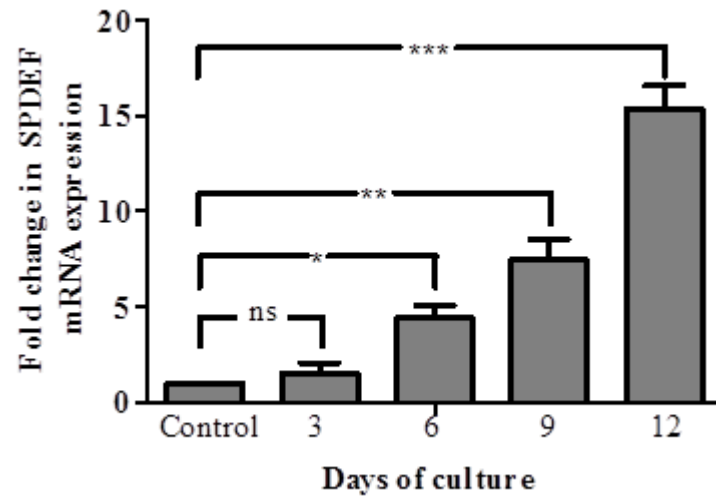
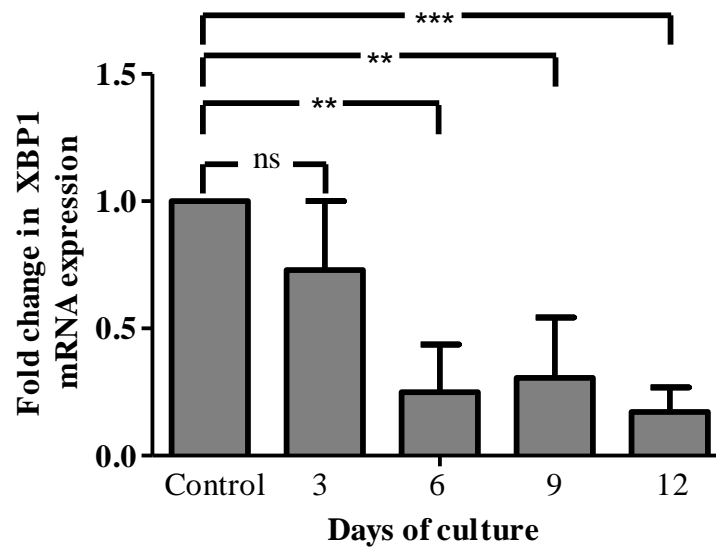
(a)**(b)**

Figure 19: Estimation of SPDEF (a) and XBP1 (b) mRNA expression in the mGS cells grown on microfibrous scaffold for 3, 6, 9, and 12 days normalised with GAPDH expression. The control bars represent level of mRNA in mGS cells grown in tissue culture plate. **= $p < 0.001$; ***= $p < 0.0001$

lectins specific for different gastric epithelial cell lineages: surface mucous or pit cells (UEAI lectin), parietal cells (DBA lectin) and gland mucous cells (GSII lectin). The results showed that the cells neither bind to UEAI nor DBA lectins, but do bind to GSII lectin as demonstrated with fluorescence microscopy (Fig. 21a) and confirmed with confocal microscopy (Figs. 21b,c). The number of cells labeled with GSII lectin was counted in 7 different images of cryosections obtained from 3 microfibrous scaffolds maintained in culture media for 9 days. Counts of the total number of cells labeled with Hoechst and those bound to GS II lectin showed that approximately 50% of the cells had differentiated into gland mucous cells. Therefore, it seems that PCL microfibrous scaffold is suitable for supporting not only growth of mGS cells but also their differentiation into gland mucous cells.

3.5 Effects of acidic pH on cultured mGS cells (Experiment 4)

Since, the long term aim of this study is to establish a model system that could have *in vivo* applications, it was necessary to know how an acidic environment comparable to that of the stomach could affect the mGS cells and the microfibrous PCL scaffold. The mGS cells were exposed acidic pH while growing in RPMI media in 2D and then 3D culture systems.

3.5.1 Effects of acidic pH on the viability of mGS cells in 2D culture

The mGS cells were cultured in 24- or 96-well plates using the usual RPMI media. On day 2, the media was replaced by fresh RPMI but its pH was adjusted at different values ranging from 3.0 to 7.4. After 5 hours incubation in the acidic pH media, fluorescence micrographs clearly showed that the cells incorporated calcein at pH 7.4 (Figs. 23g, h). However, when mGS cells were exposed to pH 3.0, they

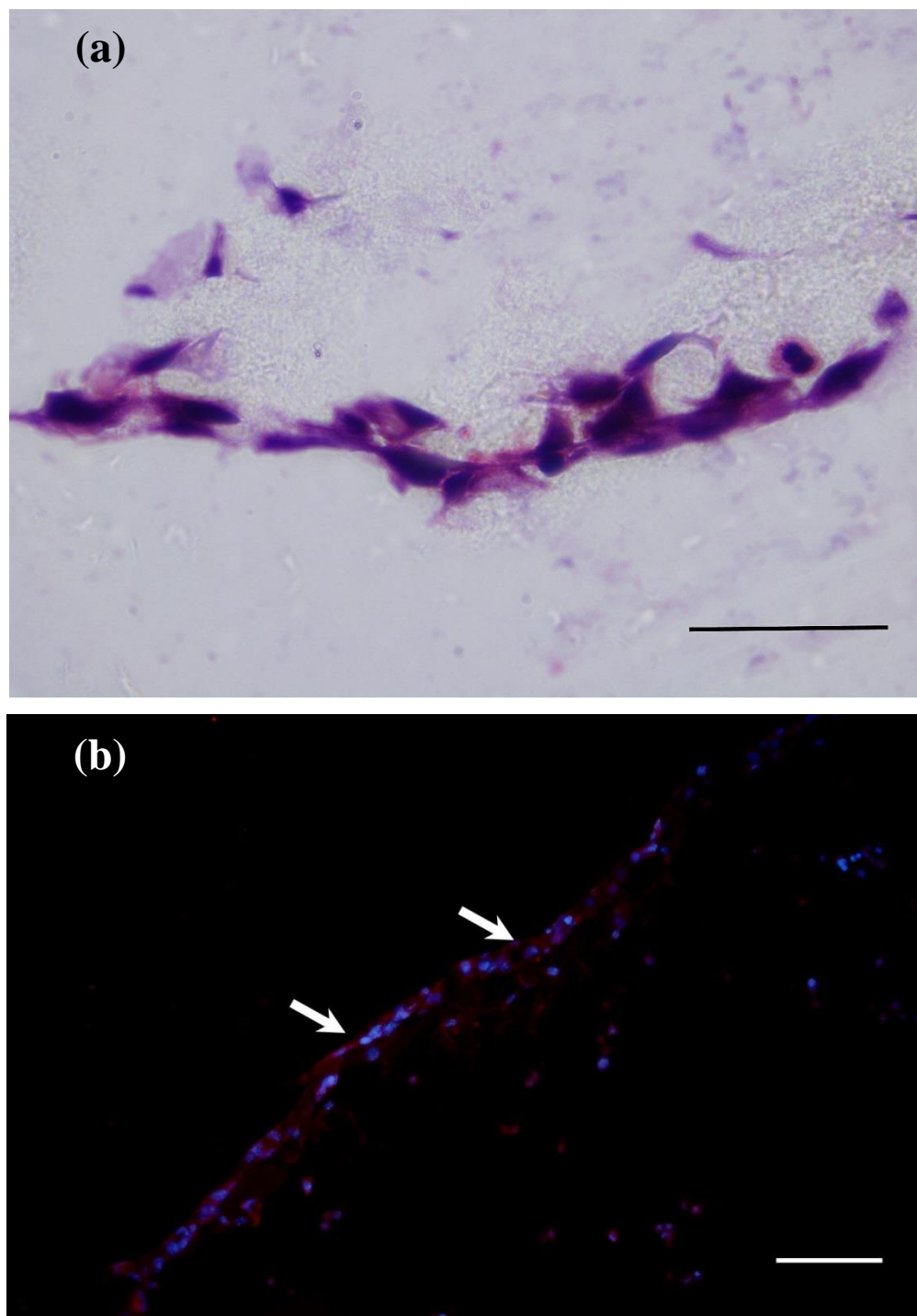


Figure 20: Microscopic analysis of cryostat section of mGS cells growing on microfibrous PCL scaffolds for 9 days. **(a)** Light micrograph of mGS cells stained with haematoxylin and eosin. Arrows are pointing to hematoxylin-stained nuclei. **(b)** Fluorescence micrograph of mGS cells probed with anti-TFF2 antibodies (red) and counter stained with Hoechst (blue). Arrows indicate TFF2-expressing cells. Bar = 50 μm (a, b)

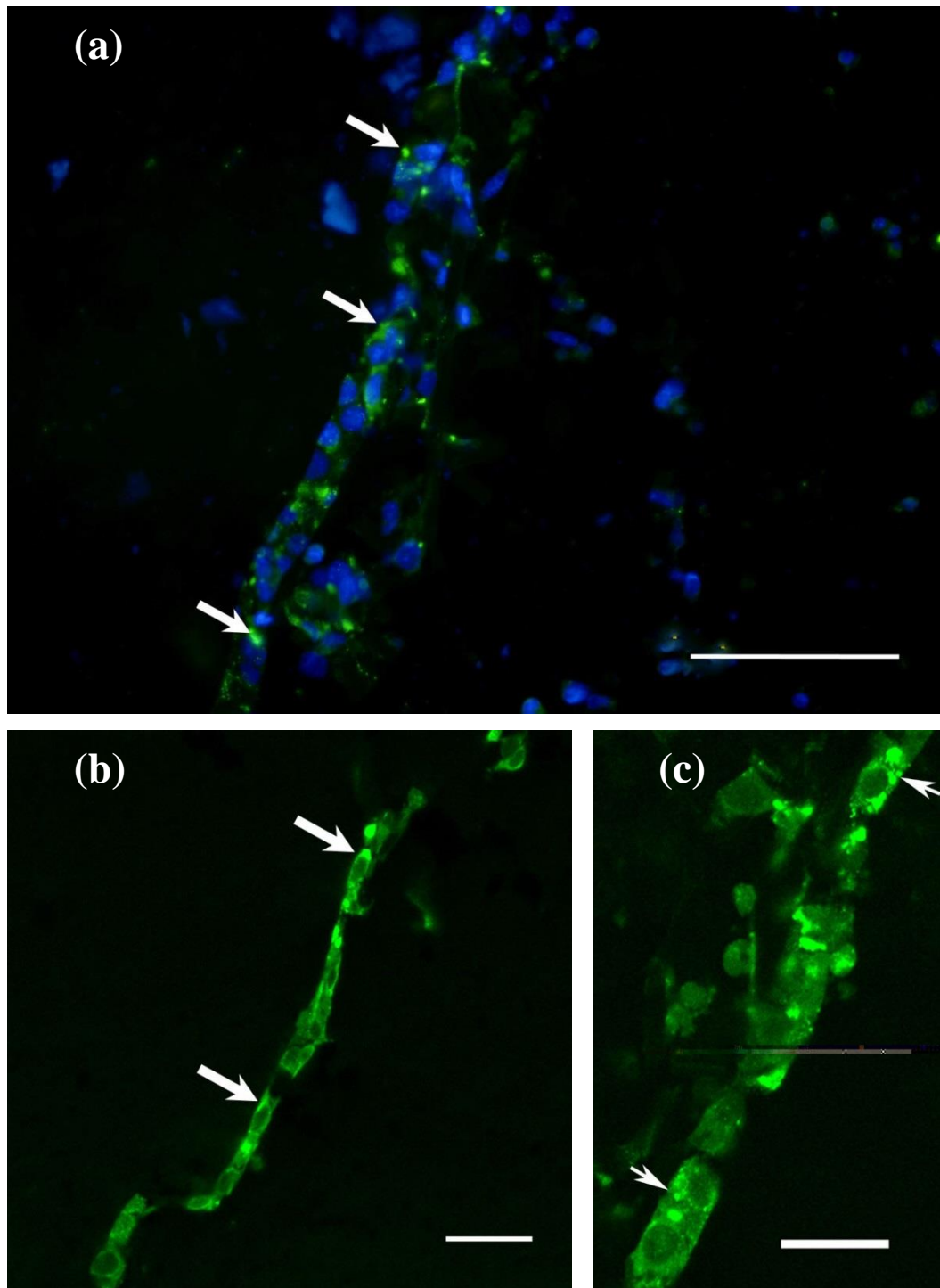


Figure 21: Lectin histochemical analysis for cryosections of mGS cells growing on microfibrinous scaffolds for 9 days. **(a)** Micrograph showing GSII (green at arrow tips) binding and Hoechst (blue) nuclear labelling. **(b, c)** Confocal micrographs confirm the GSII (green at arrow tips) binding to the cytoplasm of cultured cells. Note the granular nature of GSII-labelled areas in the cytoplasm (arrows in c). Bar = 50 μm (a, b) and 25 μm (c)

incorporated propidium iodide, and hence were all dead at pH 3.0 (Fig. 23b). Cultured mGS cells at pH 4.5 showed double labelling indicating that some cells were deteriorating and others were viable (Fig. 23d). At pH 5 and 5.5, the cells were labeled with calcein indicating their viability; but it was noted in all experiments that the adherence of the mGS cells at pH 5 was highly compromised. The cells tended to detach in sheets. At pH 5.5, cell viability was significantly good without affecting its adherence (Figs. 23e, f, 22a) when compared to other low pH values. The number of dead cells incorporating propidium iodide at pH 4.5 to 5.5 were significant ($***=P<0.0001$), whereas cell death at pH 5.5, 6.0 and 7.4 was not significant (Fig. 22b).

3.5.2 Effects of acidic pH on the migration of mGS cells in 2D culture

To test whether the growth of mGS cells in acidic environment would affect their migration and capacity to heal in case of damage, they were seeded in 6 well plates at 16,000 cells per well and after reaching semi-confluence (2 days), a linear scratch was made in the center of the wells using the tip of a 1-ml pipette. The cells were then washed with PBS and incubated with 10% RPMI media for 1 hr to recover from the induced scratch or wound. The normal media was replaced with 10% RPMI at pH 6.0. Scratched cells in control wells were grown in RPMI media of pH 7.4. Cell migration to cover the denuded surface of the well was examined in micrographs taken at the same magnification (10X) after 1 hr and 1-3 days (Fig. 24). The width of the wound was estimated in all wells at all time-points. The results clearly showed a significant difference between the wound widths in case of cells cultured in pH 6.0 when compared to control (pH 7.4) at different time-points (Fig. 25). At pH 7.4, the wound area is gradually covered by the migration of cells after 1

and 2 days. By 3 days of culture, the wound area was almost completely disappeared. However, at pH 6.0, the migration of mGS cells was very slow at all time-point (Figs. 24, 25).

3.5.3 Effects of acidic pH on the mechanical properties of microfibrinous PCL scaffolds

The sheets of microfibrinous scaffolds prepared by the electrospinning technique were approximately 0.9 mm in thickness. They appeared as a complex meshwork of microfibers which were variable in diameter, 8-20 microns (Fig. 26a). Moreover, high magnification SEM micrographs clearly revealed the interconnected fibers and its random arrangement (Fig. 26b).

Tensile testing on the microfibrinous scaffold samples exposed to RPMI media of pH 3.0, 5.5, and 7.4 for 3, 6, 9, and 12 days showed higher peak stress as compared to mouse stomach value (Fig. 27). Therefore, the acidic environment had a considerable effect on the stability of the microfibrinous scaffold. Increasing the incubation time of the scaffold and the acidity values were associated with reduction in peak stress of the scaffold. At day 3, for pH 7.4, the peak stress was 0.7 MPa. In case of pH 3.0, the peak stress was reduced to 0.52 MPa. By reaching 9 days of exposure to pH 7.4, there was no much significant change in the peak stress whereas in the case of pH 3.0 it became 0.22 MPa which was still above the peak stress of mouse stomach (0.18Mpa). Microfibrinous scaffold at pH 5.5 showed a peak stress of 0.62 MPa at day3 and 0.54 MPa by day 9.

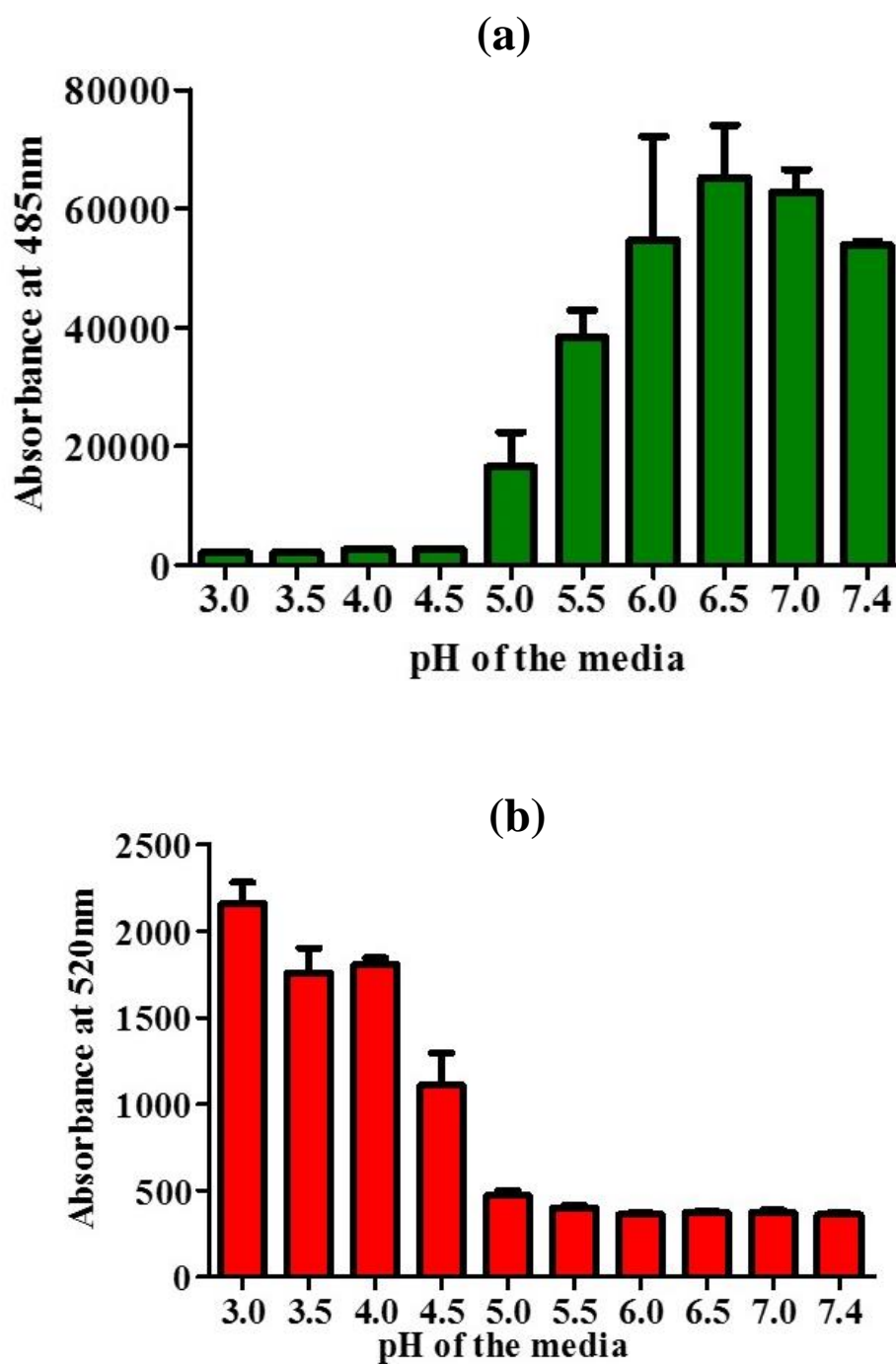


Figure 22: Cell viability and death assay of mGS cells cultured in RPMI at different pH values and incubated with calcein (a) and propidium iodide (b). Fluorometric measurements were carried out for calcein (a) and propidium iodide (b) uptake by living and dead cells at the absorbance of 485 and 520 nm, respectively.

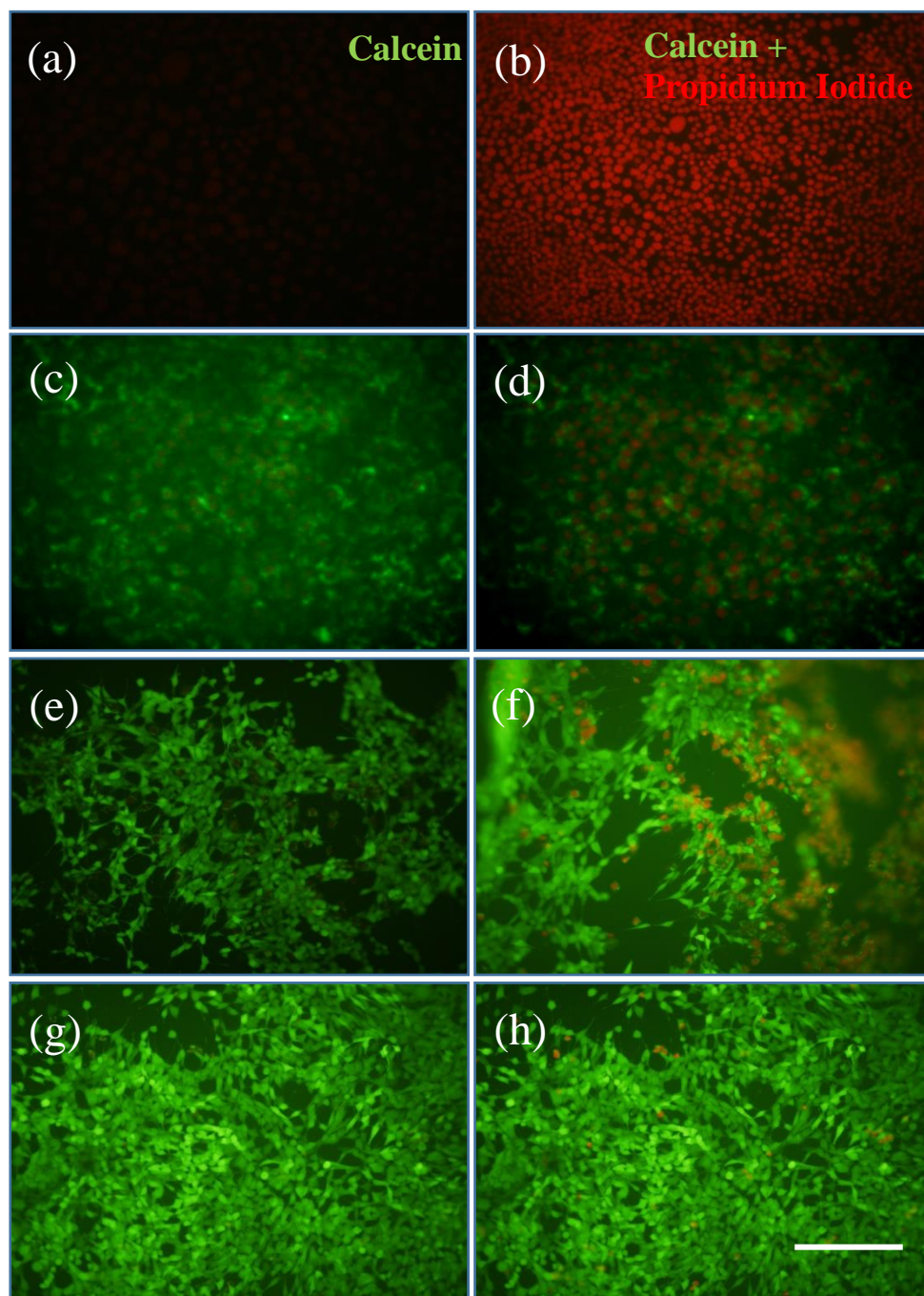


Figure 23: Fluorescence micrographs of calcein (a,c,e,g) and double calcein-propidium iodide (b,d,f,h) labeling of mGS cells cultured for 2 days in normal RPMI and then for 5 hours in RPMI media at pH values of 3.0 (a, b), 4.5 (c, d), 5.5 (e, f), and 7.4 (g, h). Scale bar = 100 μ m.

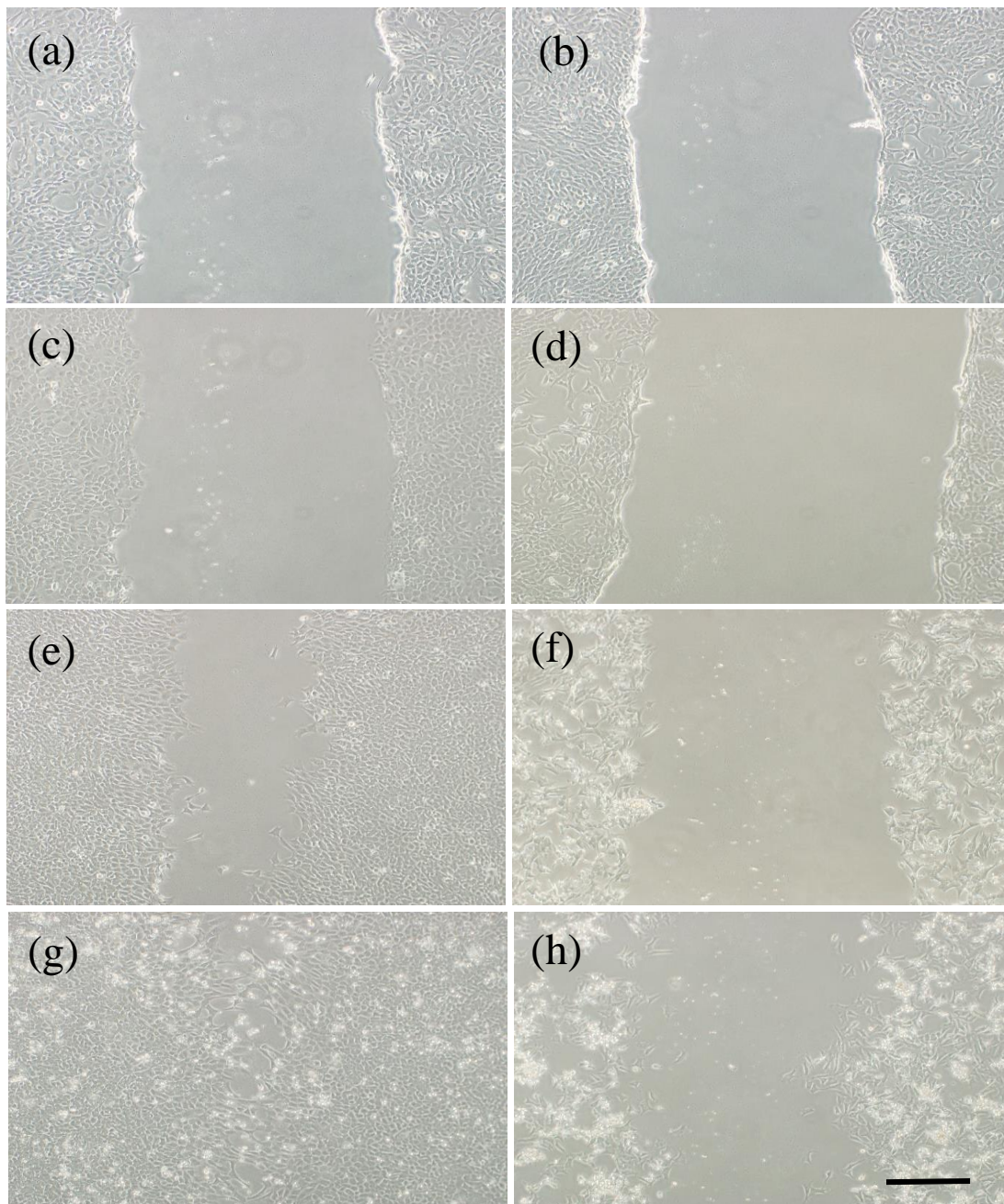


Figure 24: Phase contrast microscopic images of wounded monolayers of mGS cells incubated in RPMI media at pH 7.4 (a,c,e,g) and 6.0 (b,d,f,h) for 1hr (a, b), 1day (c, d), 2 days (e, f), and 3 days (g,h). Scale bar = 200 μ m.

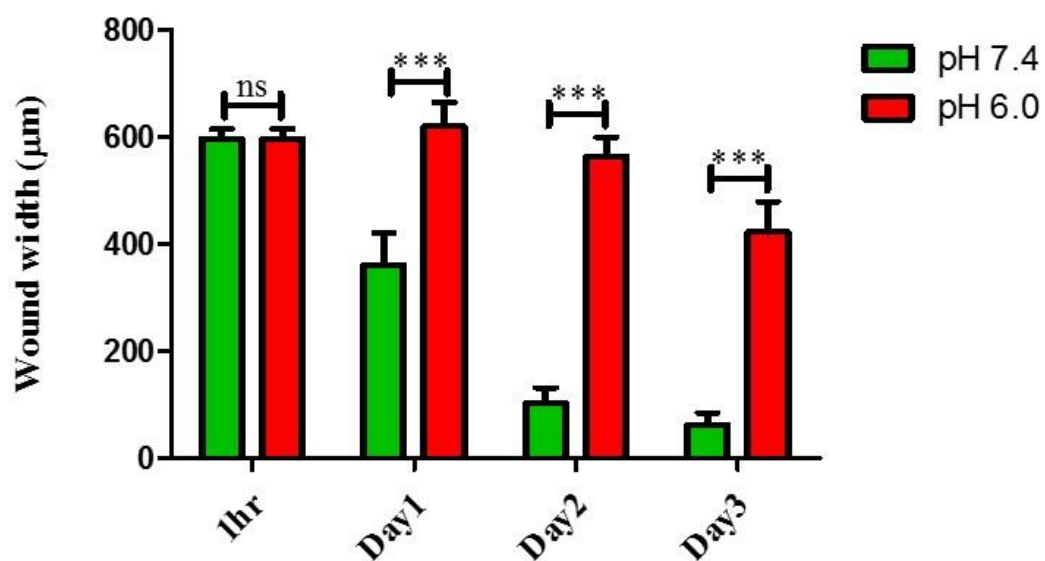


Figure 25: In vitro wound healing assay. Measurements of the widths of the wounds induced in mGS cells cultured for 1h and for 1 to 3 days in RPMI media at pH 7.4 and 6.0. The data are presented as the mean \pm SD. *** = $p < 0.0001$

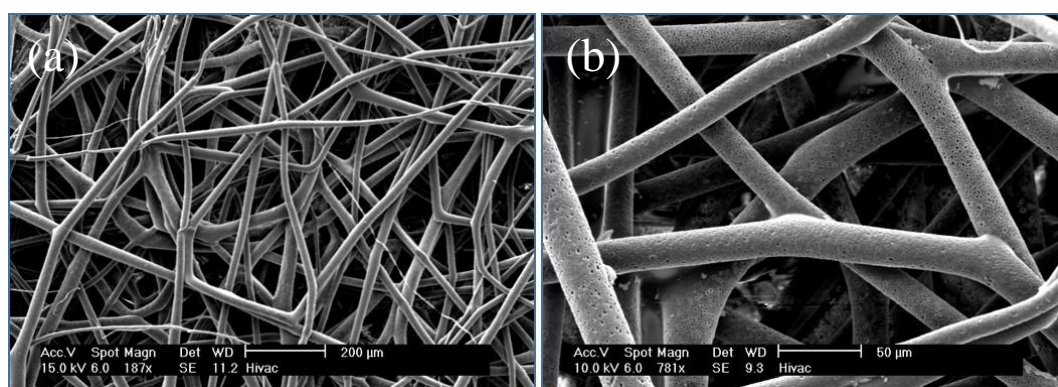


Figure 26: Scanning electron micrographs of microfibrillar PCL scaffolds showing their surface topography at low (a) and high (b) magnifications. (a) Note the random arrangement of microfibrils Bar = 200 μm . (b) Note the variable diameters and porosity of the interconnected microfibrils. Bar = 50 μm

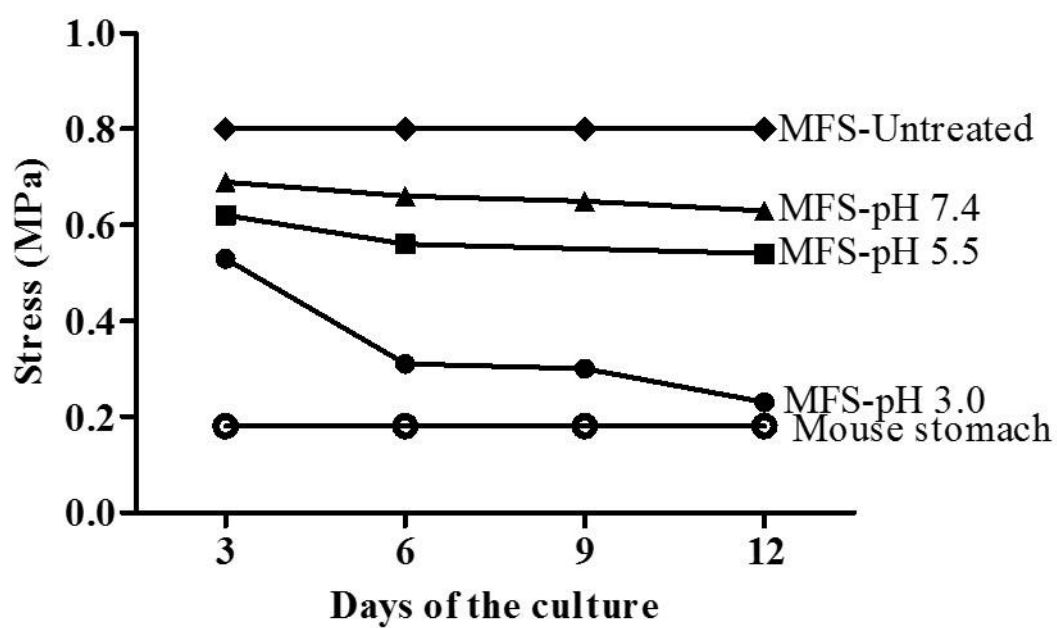


Figure 27: Measurements of stress of the microfibrous PCL scaffolds incubated for 3, 6, 9, and 12 days in RPMI media at pH 3, 5.5, and 7.4. The stress obtained was compared to dry (untreated) microfibrous PCL scaffold and mouse stomach tissue.

3.5.4 Effects of acidic pH on the chemical properties of microfibrinous PCL scaffolds

Figure 28 shows FTIR spectra of pure PCL scaffold as well as scaffolds treated at pH 3.0, 5.5 and 7.4 for 12 days. The similarity between the spectra of all samples indicates the structural stability of PCL scaffolds where no evidence of degradation products was found despite the acidic pH of the culture media. It should be mentioned that PCL degrades over a course of 2 years. However, it was expected that degradation could be enhanced by the high surface area of the microfibers and the acidification of the media. The current results showed that, in acidic environment, microfibrinous PCL scaffolds maintain their structural integrity without degradation and, therefore, could be useful for implantation in the wall of the stomach *in vivo*.

3.5.5 Effects of acidic pH on the viability of mGS cells cultured on microfibrinous PCL scaffolds

To determine the pH value that the 3D culture system can tolerate, mGS cells were first seeded on microfibrinous PCL scaffolds using regular RPMI media (pH 7.4) for 2 days. Then the media was changed with fresh RPMI at pH values 7.4, 5.5, and 3. At pH 3.0, there was a significant increase in the number of dead cells stained with propidium iodide, $**=p<0.001$ (Figs. 29a,b;30b). However, at pH values of 5.5 and 7.4, there were a large number of viable cells which converted the non-fluorescent calcein acetoxymethyl ester into the fluorescent compound calcein and a small amount of dead cells which were stained with propidium iodide (Figs. 29c-f, 30a). Quantification showed that changing the pH from 3.0 to 5.5 induced a highly

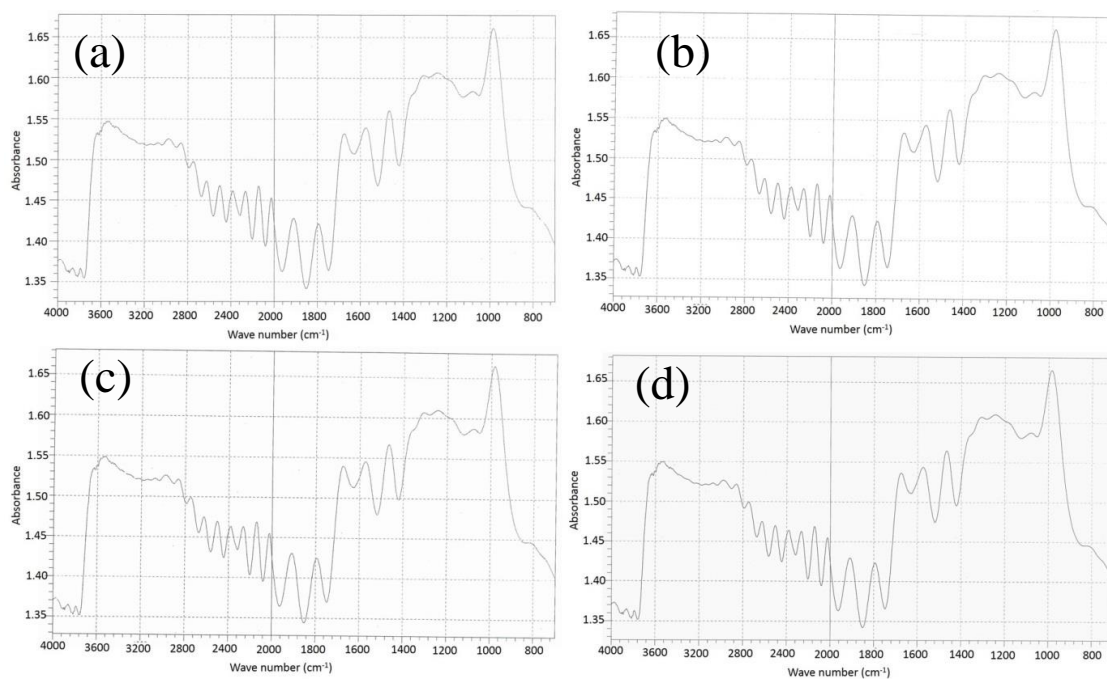


Figure 28: Infrared spectroscopy analysis of untreated PCL microfibrous scaffold (a) and PCL microfibrous scaffold samples incubated at the pH 3.0 (b), 5.5 (c) and 7.4 (d) for 12 days. The graph shows no change in the peak formation on each samples and no signs of any degradation.

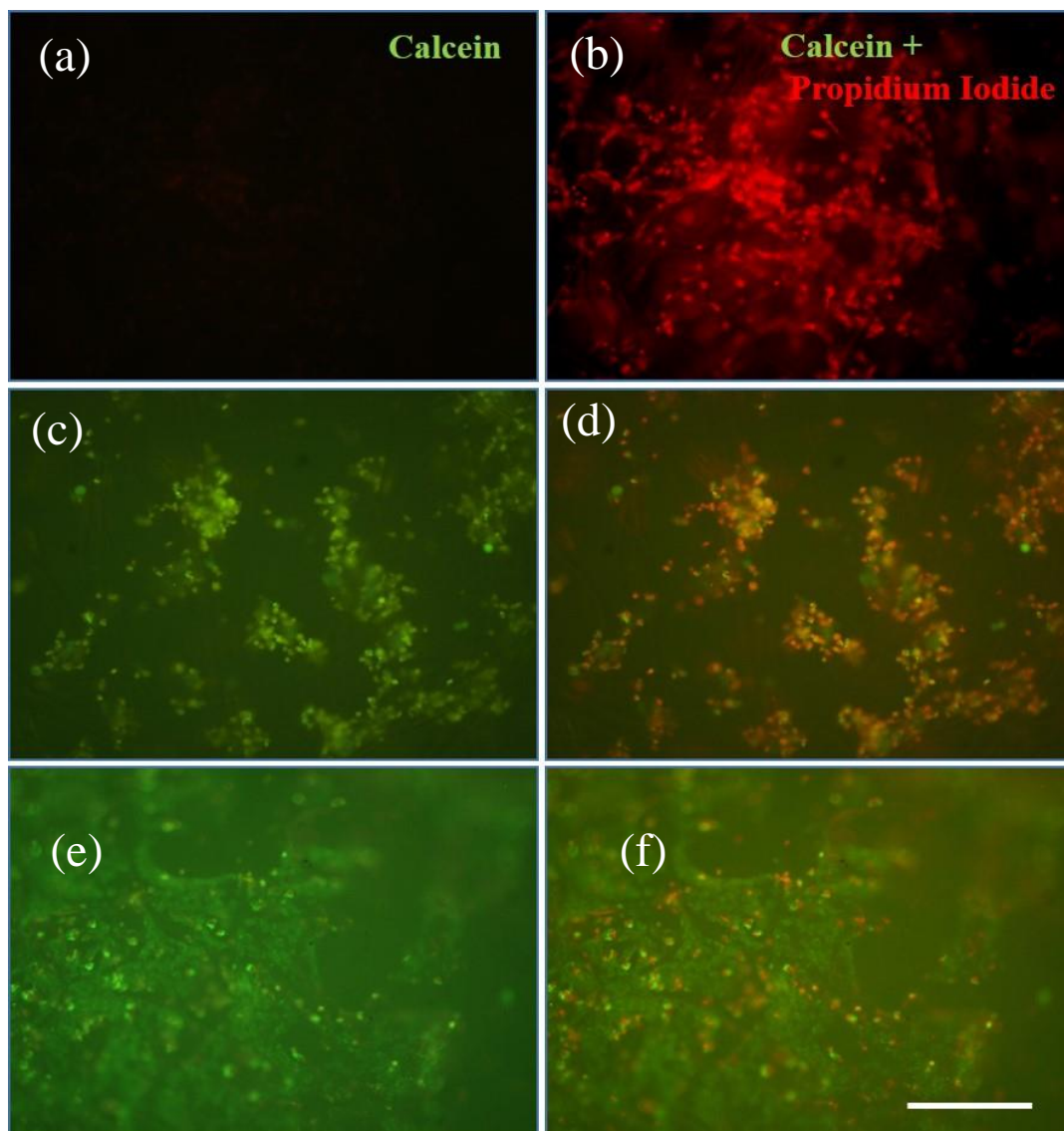


Figure 29: Fluorescence micrographs of calcein (a, c, e) and calcein plus propidium iodide (b, d, f) labeling of mGS cells grown on microfibrous PCL scaffolds for 2 days in RPMI media at pH 7.4 and then for 3 hours in RPMI media of pH 3.0 (a, b), 5.5 (c, d) and 7.4 (e, f). Bar = 100 μ m.

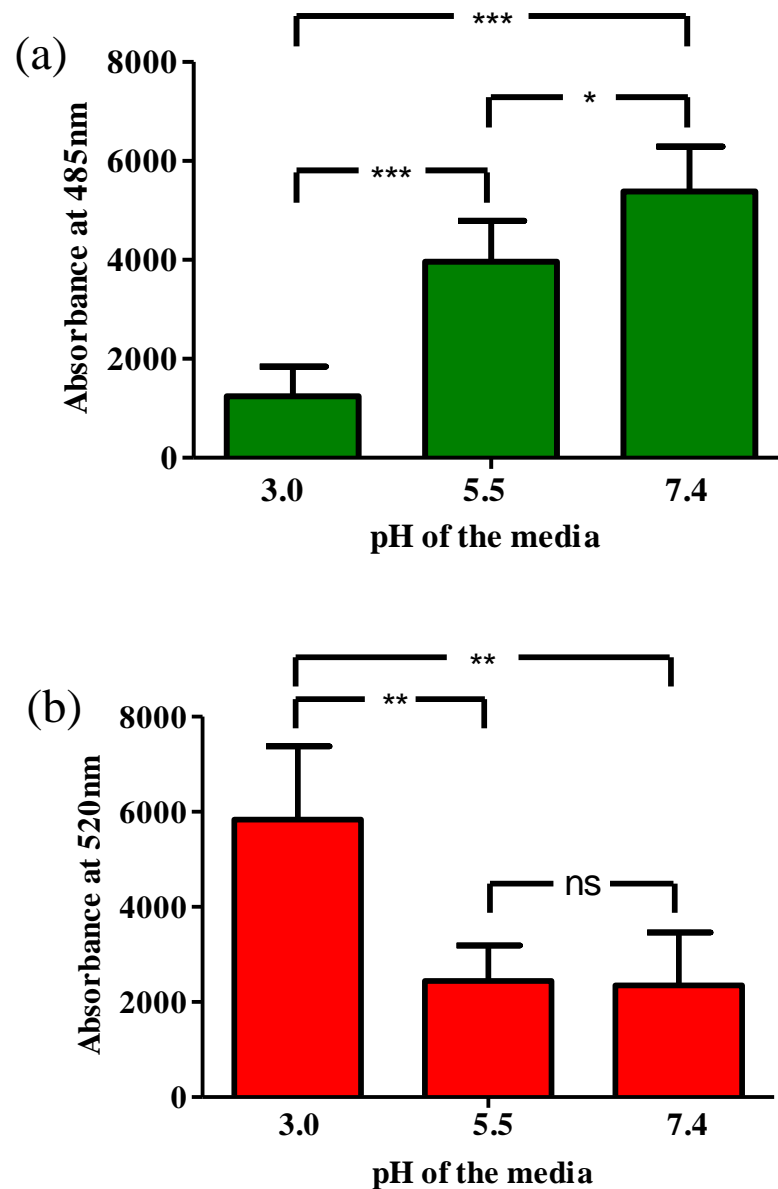


Figure 30: Cell viability and death assay of mouse gastric stem cells grown on microfibrous PCL scaffolds using RPMI at pH values of 3.0, 5.5, and 7.4. (a) Calcein uptake by living cells was measured at 485 nm. The cell viability was significantly high at pH 5.5 and 7.4 compared to that of pH 3.0. (**= $p < 0.0001$) and the difference between viability of cells cultured at pH 5.5 and 7.4 are less significant (*= $p < 0.05$). (b) Propidium iodide uptake by dead cells on microfibrous scaffold measured at the absorbance at 520 nm. The cell death showed significant difference at pH 5.5 and 7.4 compared to that of pH 3.0 (**= $p < 0.001$) and the difference between the number of dead cells at pH 5.5 and 7.4 was not significant

significant increase ($***=p<0.001$) in the cell viability (Fig. 30a). The difference in viability of mGS cells cultured at pH 5.5 and 7.4 was also significant, $=p<0.05$ (Fig. 30a). Measurement of cell death using propidium iodide incorporation showed no significant change in the number of dead cells when the pH of the media was changed from 7.4 to 5.5. However, there was a significant increase in the number of dead cells at pH 3.0, $**=p<0.001$ (Fig. 30b).

3.5.6 Effects of acidic pH on gene expression levels of mGS cells seeded in culture plates (2D) and on microfibrous PCL scaffolds (3D)

To test the effect of acidic pH in 2D culture, the mGS cells grown on tissue culture plate with 10% RPMI at pH 5.5 for 3 and 9 days were compared with those grown at pH 7.4. The RNA was extracted and utilized for the generation of cDNA using reverse transcription assay. Quantitative PCR was then applied using primers specific for cell proliferation (PCNA) and stem cell signaling (DCLK1) genes. The results showed that the acidic pH induced up-regulation in the mRNA expression level of DCLK1 and down-regulation of PCNA expression (Figs. 31, 32).

In 3D culture, while growth of mGS cells on microfibrous PCL scaffolds for 3, 6, 9 and 12 days at pH 7.4 showed a gradual increase in the expression levels of DCLK1 (Fig. 18b), the acidic pH together with 3D culture demonstrated an enhancement in the up-regulation of the DCLK1 expression after 3-day culture (Fig. 33). The proliferation marker PCNA showed a significant down-regulation in both 2D (Fig. 32) and 3D (Fig. 34,18c) culture systems except for the up-regulation noticed in cultured mGS cells on the scaffolds at pH 5.5 for 3 days (Fig. 34).

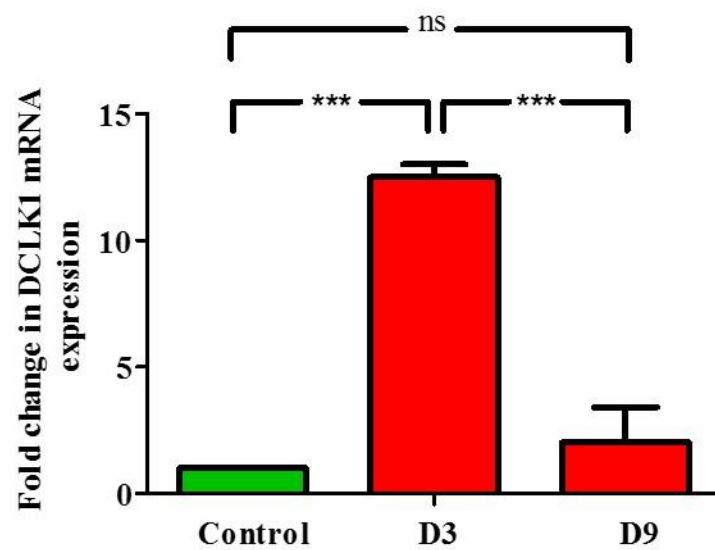


Figure 31: The mRNA expression of DCLK1 in mGS cells grown in 2D culture plates at pH 5.5 for 3 and 9 days as compared to control cells growing at pH 7.4. Values were normalized to GAPDH and the values of day 3 and 9 were compared to control sample which was normalized to 1. Note that DCLK1 expression is significantly increased on day 3 at pH 5.5 (**= $p < 0.0001$) whereas on day 9 the level of expression is not significant when compared to control, but significant when compared to that of day 3.

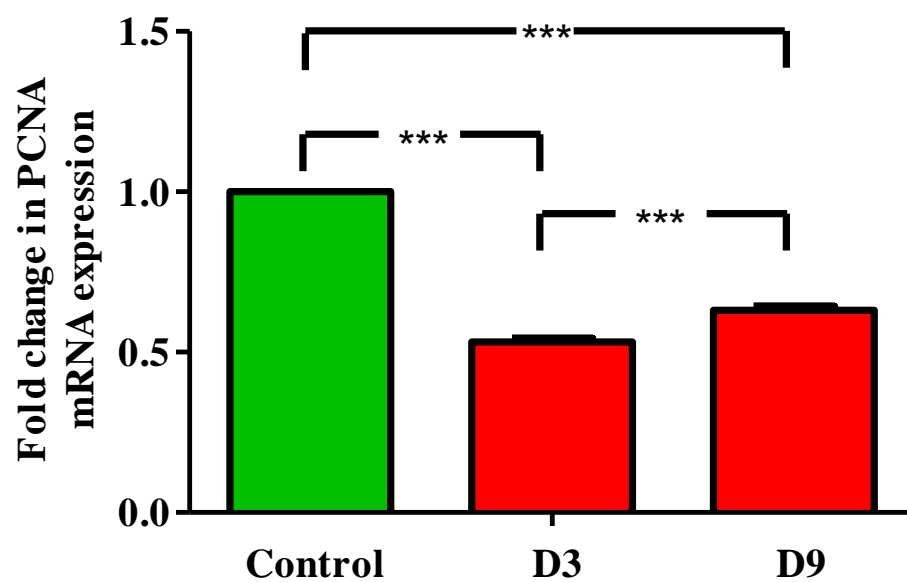


Figure 32: The mRNA expression of PCNA in mGS cells grown in 2D culture at pH 5.5 for 3 and 9 days. Note that PCNA mRNA expression is significantly down regulated in days 3 and 9 (***= $p < 0.0001$).

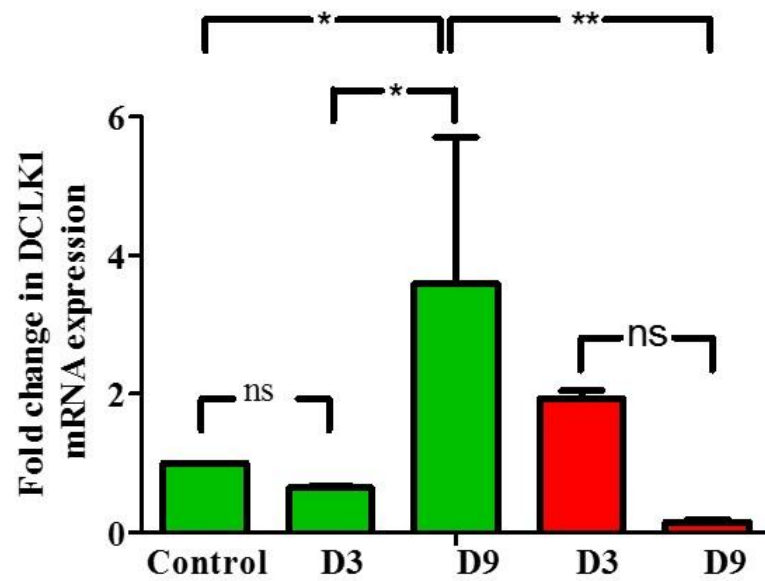


Figure 33: The expression of DCLK1 mRNA in mGS cells grown in 3D culture incubated at pH 5.5 and 7.4 for 3 and 9 days. DCLK1 expression up regulation on day9 pH 7.4 as well as the difference between day3 and day9 pH 7.4 grown cells on PCL microfibrous scaffold were less significant ($*=p<0.05$). While mRNA expression is highly significant between day pH5.5 and 7.4 grown cells on PCL microfibrous scaffold ($**=p<0.0092$).

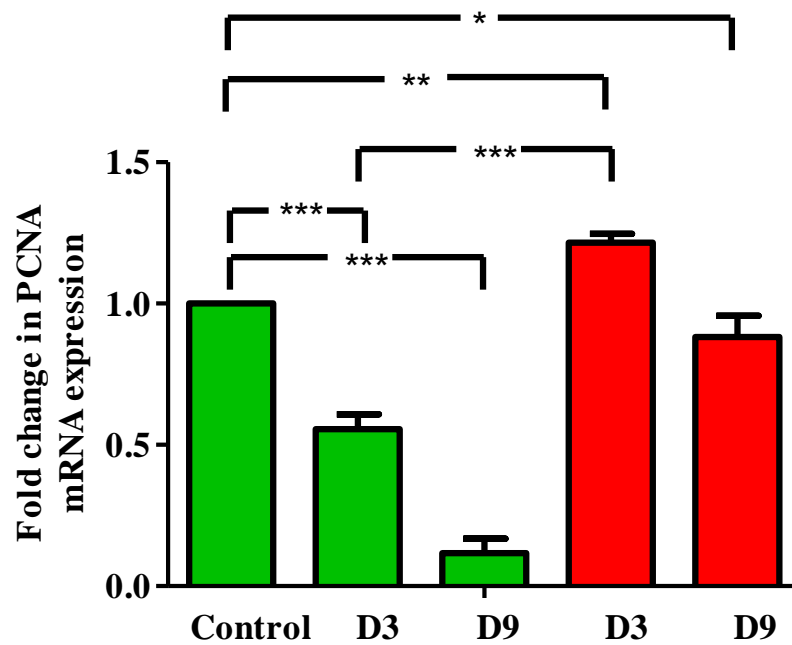


Figure 34: The mRNA expression of PCNA in mGS cells grown on 3D (PCL microfibrous scaffold) cell culture incubated at pH 5.5 and 7.4 for 3 and 9 days. PCNA mRNA expression was down regulated on microfibrous scaffold on day 3 and 9 at pH7.4 significantly ($***=p<0.0001$). But on pH 5.5, the PCNA mRNA expression were up regulated on day3 ($**=p<0.001$) and day9 ($=p<0.05$).

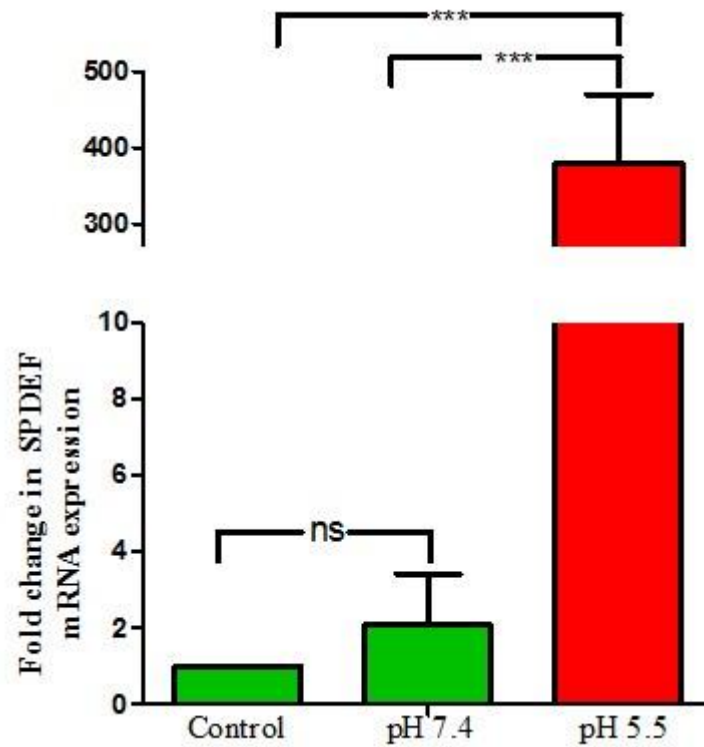


Figure 35: Expression of the mRNA of the transcription factor SPDEF in mGS cells grown on 3D (microfibrous) PCL scaffolds and incubated at pH 5.5 and 7.4 for 3 days. The amount of SPDEF mRNA were up-regulated on day 3 at pH 5.5 and when compared to control cells the level of increase was significant (**= $p < 0.0001$).

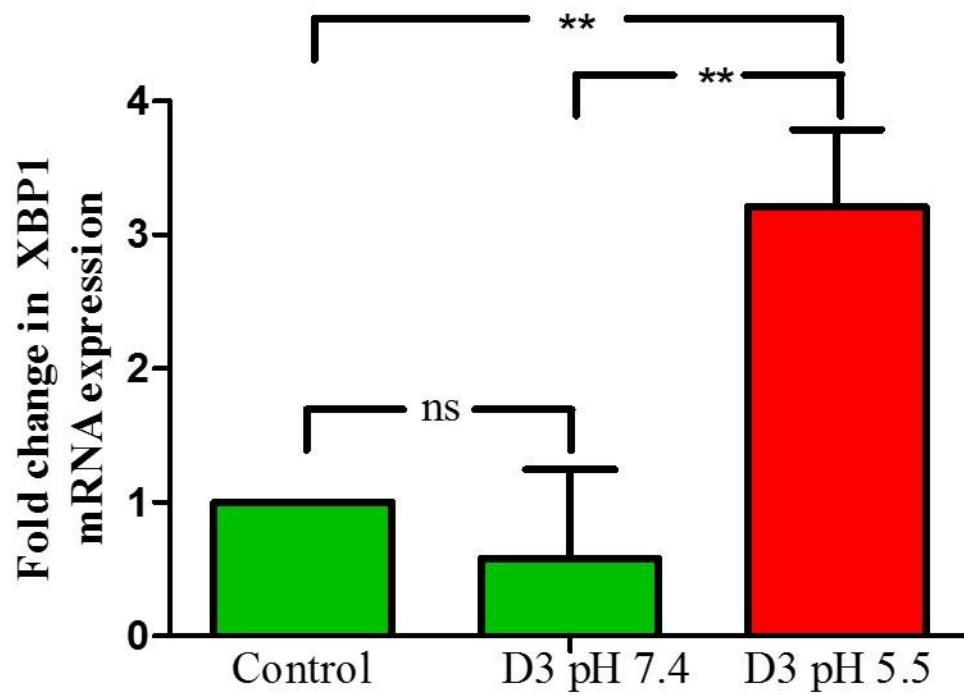


Figure 36: Expression of the mRNA of the transcription factor XBP1 in mGS cells grown on 3D (microfibrous) PCL scaffolds and incubated at pH 5.5 and 7.4 for 3 days. The amount of XBP1 mRNA were up-regulated on day 3 at pH 5.5 and when compared to control cells the level of increase was significant (**= $p < 0.001$).

In order to check whether the acidic environment has also affected the expression of the transcription factor SPDEF and XBP1 involved in the differentiation of gastric gland mucous cells, total RNA extracted from mGS cells was processed for qRT-PCR and using SPDEF primers. Interestingly, the results showed an upregulation in the expression level of SPDEF and XBP1 only after 3 days of 3D culture at pH 5.5 (Fig.35,36).

3.5.7 Effects of acidic pH on the lectin- and immuno-cytochemical localization of gastric epithelial biomarkers in mGS cells seeded on microfibrinous PCL scaffolds

The mGS cells were grown on microfibrinous scaffolds for 3 days using RPMI media at pH 5.5 and 7.4 and processed for cryosectioning. Some sections were stained for H&E for general histology and orientation. Adjacent sections were probed using gastric epithelial biomarkers, namely fluorophore-conjugated GSII, UEA, and DBA lectins as well as primary antibodies specific for TFF1, TFF2, H,K-ATPase β subunit. The results showed that mGS cells grown in normal pH 7.4 on 3D scaffold for 3 days did not bind to any of the lectins or antibodies used. However, several mGS cells cultured on scaffolds for 3 days at pH 5.5 were positively stained with GSII lectin (Fig. 37). This indicated that the acidic pH of the RPMI media did not interfere with the differentiation of mGS cells into gland mucous cells. Moreover, these data indicated that the acidic environment induced precocious differentiation of mGS cells into gland mucous cells which appeared only after 3-day culture (not after 9-day culture as in the normal culture conditions).

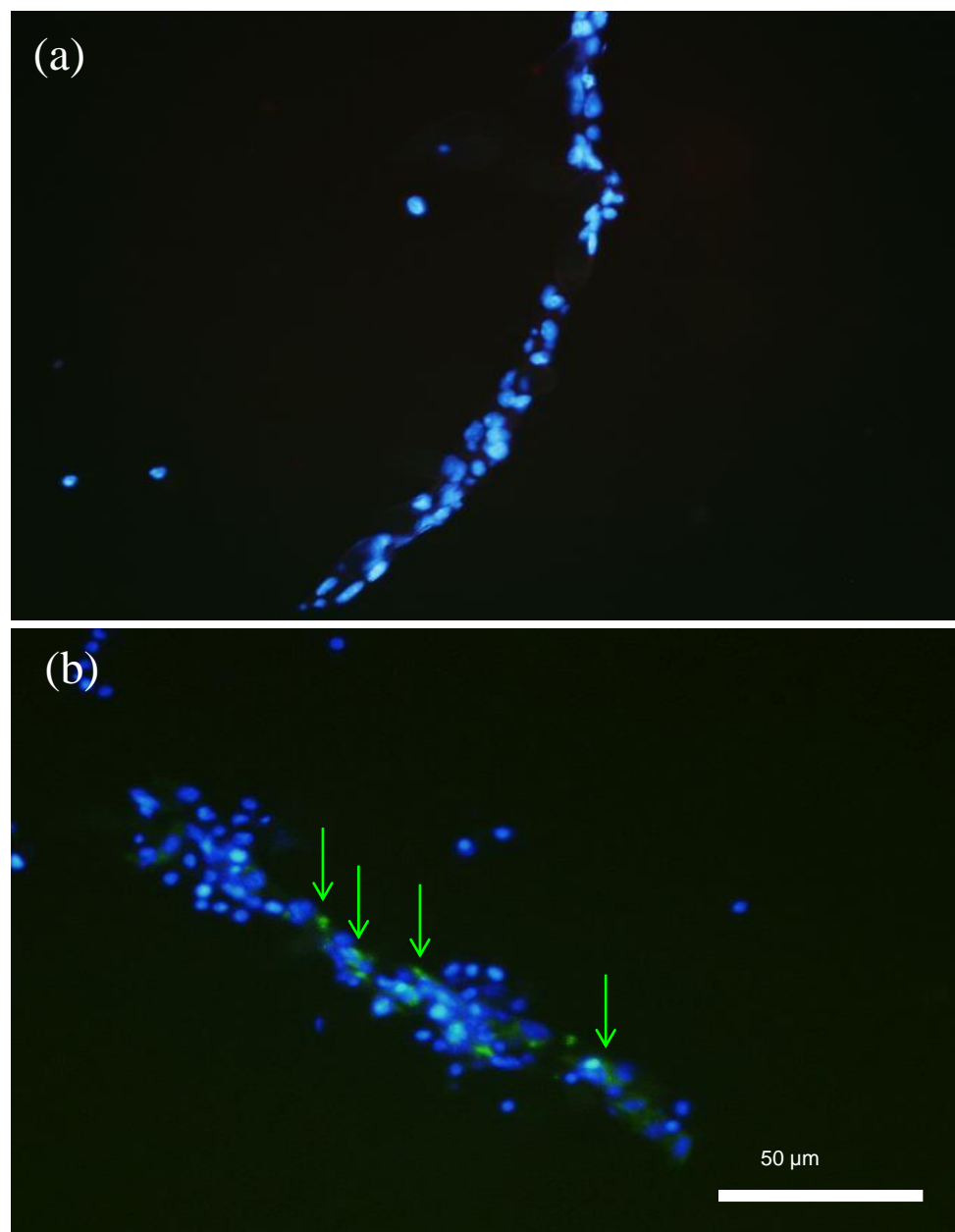


Figure 37: Fluorescence micrographs of mGS cells growing on microfibrinous PCL scaffolds for 3 days using RPMI media at pH values of 7.4 (a) and 5.5 (b) and probed with DAPI (blue) and GSII (green). Note that at pH 7.4, while all nuclei are labeled with DAPI, there is no GSII binding. At pH 5.5, the GSII (green) binding is shown in several cells (arrows). Scale bar = 50 μm .

Chapter 4: Discussion

This study describes an *in vitro* model system for the growth of mGS cells on synthetic biodegradable scaffolds that support their differentiation into glandular mucous cells. This model system is a step forward in establishing a method for engineering gastric mucosal tissue that could have future applications in regenerative treatment of gastric cancer/ulcer patients undergoing gastrectomy. Since complete or even partial loss of the stomach may lead to devastating and life-threatening consequences, the long term plan of this research is to provide the basis for autologous or syngeneic transplantation of engineered gastric tissues using gastric stem cells.

Adult stem cells have already shown promise for tissue engineering application but it is important to characterize the culture conditions, properties of the scaffold platforms and the growth of the seeded cells that would result in a new functional tissue (Soleimani et al., 2010; Jaklenec et al., 2012). Such *in vitro* model could also serve to provide a platform to study growth and differentiation programs of stem cells and to serve as a useful model to study the effects of chemotherapy or newly developed drugs or compounds on stem cells and mucous cell differentiation.

4.1 Topographical properties of microfibrinous PCL scaffolds suggest their suitability for mGS cell growth

In the present study, the surface topography of the prepared three types of scaffolds was revealed using SEM. The differences in the surface roughness of the nonporous scaffold as well as the number and size of pores in microporous and

microfibrinous scaffolds could contribute to the differences in their mechanical properties.

Several studies have demonstrated the role of surface topography and porosity of scaffolds on adhesion, growth, and differentiation of cultured cells. Changing surface topography of polyvinyl alcohol surfaces by inducing abrasions was found to improve orientation and elongation of fibroblasts and cardiomyocytes (Au et al., 2007). Generation of porous PCL scaffolds using the salt leaching method provides large surface area which was thought to improve cell adhesion (Heijkants et al., 2008). It was also found that the size of pores affect the expression of genes related to chondrogenic differentiation and cell attachment (Wang et al., 2010). Recently it has been shown that seeding of human retinal pigment epithelial cells on porous PCL wells significantly improves cell density, pigmentation, barrier function, up-regulation of specific genes, and polarized growth factor secretion (McHugh et al., 2014). In addition, when fetal pigment epithelial cells were grown on electrospun PCL scaffolds, they showed the highest cell densities, deeper pigmentation, and more uniform hexagonal tight junctions (Liu et al., 2014).

Although a number of scaffolds have been manufactured and utilized for cell growth, electrospun fibrous scaffolds remain attractive due to their high surface area-to-volume ratio, porosity, and 3D architecture. Previous studies showed the potential of PCL fibers to support growth of periodontal ligament cells which display mesenchymal stem cell properties (proliferation and osteogenic differentiation). In another study, human mesenchymal stem cells demonstrated similar high osteogenic differentiation on PCL with surface modification and in presence of pulsed electric field (Hess et al., 2012; Kim et al., 2014). Oligodendrocyte precursor cells grown on electrospun PCL scaffold induced their differentiation (Li et al., 2014). Some studies

showed that the fiber diameter could influence cell function and behavior on the scaffold (Badami et al., 2006; Sun et al., 2007; Christopherson et al., 2009; Liu et al., 2009; Yao et al., 2009; Daud et al., 2012). Porosity is also an important for transport of nutrients and metabolites. Interconnected pores are needed for the transfer of metabolites, nutrients, wastes and oxygen into the cells (Freed et al., 2006; Pham et al., 2006).

In the present study, the growth of mGS cells on the surface of PCL scaffolds with different morphologies was first evaluated. The PCL material was chosen in this study because it is a well-known biodegradable polymer that has long been used in tissue engineering (Kweon et al., 2003; Williams et al., 2005; Woodruff and Hutmacher, 2010). On equal seeding of mGS cells on different forms of PCL scaffolds, incubated under the same conditions, cell viability assay (Fig. 13) and toluidine blue staining (Fig. 11) revealed that the microfibrous scaffold was better for cell growth than nonporous and microporous scaffolds. Having a scaffold made of PCL in a fibrous form gives the virtue of high surface area for the cells to grow. In addition, having a non-woven fibrous scaffold of biodegradable PCL further provides interconnected porosity for cells to integrate and eventually form organized tissue.

4.2 Mechanical properties of microfibrous PCL scaffolds suggest their suitability for mGS cell growth

In the present study, mechanical testing of the prepared nonporous, microporous, and microfibrous PCL scaffolds showed different properties. Measurements of maximal stress and strain confirmed that the highest flexibility was achieved by microfibrous scaffold (0.35 MPa and 150%) in comparison to nonporous (8 MPa and 45%) and microporous (3 MPa and 35%) scaffolds (Figs. 9a-c, 38a).

The microfibrinous nature of scaffolds provided the maximal elongation and elasticity while testing (Fig. 8). When the same mechanical testing was applied to the mouse stomach, the values obtained for the maximal stress and strain were 0.17 MPa and 150%.

Previous studies reported that the values of maximal stress and destructive strain for human stomach specimens were 0.5-0.7 MPa and 190%, respectively (Egorov et al., 2002).

These values were very close to those obtained in the present study for the microfibrinous scaffold which were 0.35 MPa and 150%. At the same time, the highest similarity to the peak stress and strain of mouse stomach samples were also those of the microfibrinous scaffold (Figs. 9c, 38b). The values of microfibrinous samples were also in the range of stress and strain reported for human stomach samples. On a fibrous scaffold, the cells grow along the fibers and the fibers direct the growth of each cell towards each other. This forms a kind of meshwork and mimics the extracellular matrix and favors the use of fibrous scaffold for regenerative purposes (Ma et al., 2000).

4.3 Microfibrinous PCL scaffolds are suitable for mGS cell growth

In this study, both mechanical and topographical factors suggested that the microfibrinous scaffolds have more influence on cell growth and behavior. To further confirm this observation, the cell viability assays were conducted and the data obtained were compared between the three different types of scaffolds.

By using different cell viability assays and DNA quantification method, it was possible to demonstrate and confirm preferential growth of mGS cells on microfibrinous scaffolds. Calcein cell viability assay shows that microfibrinous scaffold

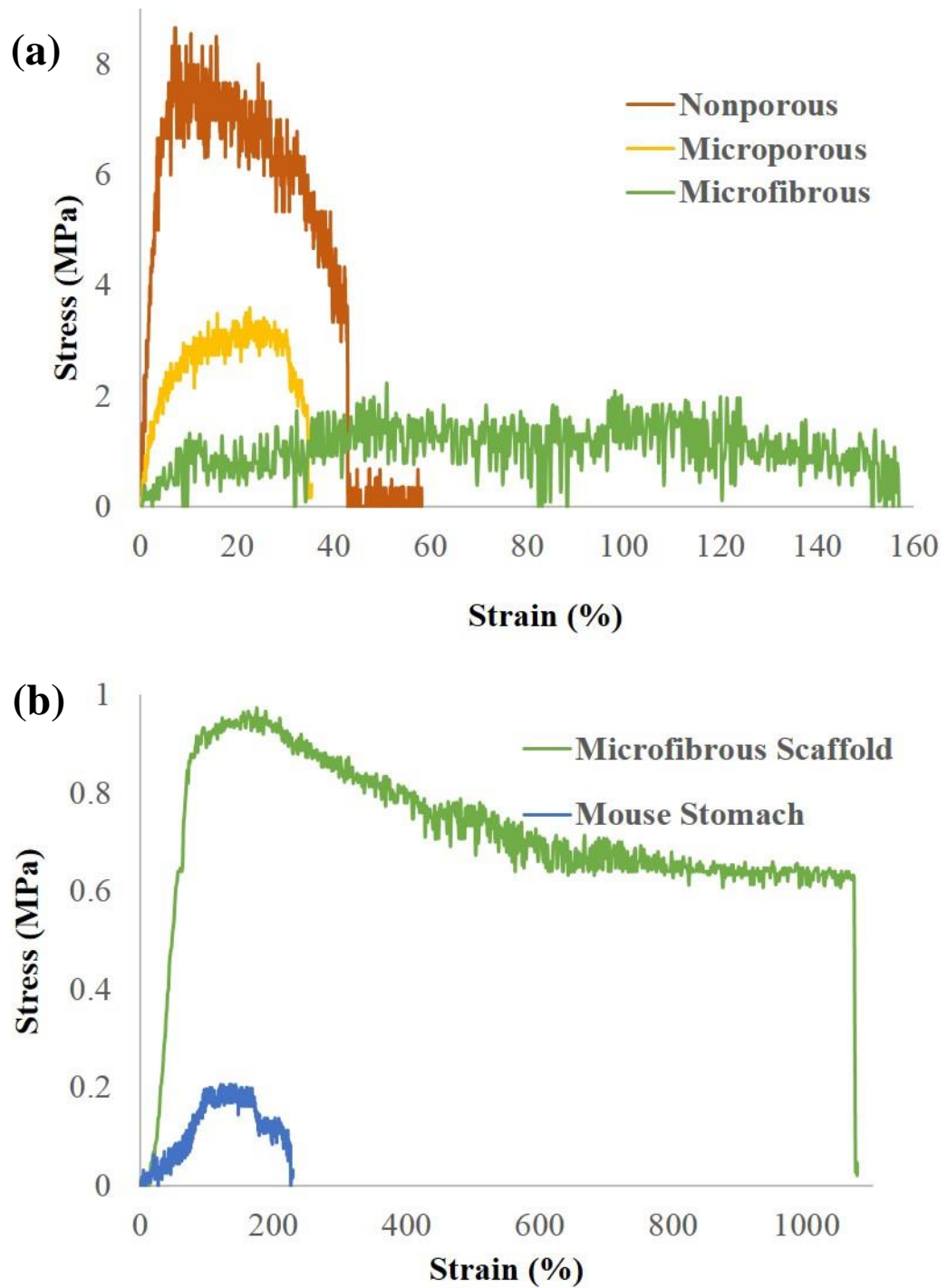


Figure 38: Comparison of the tensile curves of nonporous, microporous, and microfibrillar PCL scaffold samples (a) and comparison of the tensile curves of the mouse stomach wall with the microfibrillar samples (b)

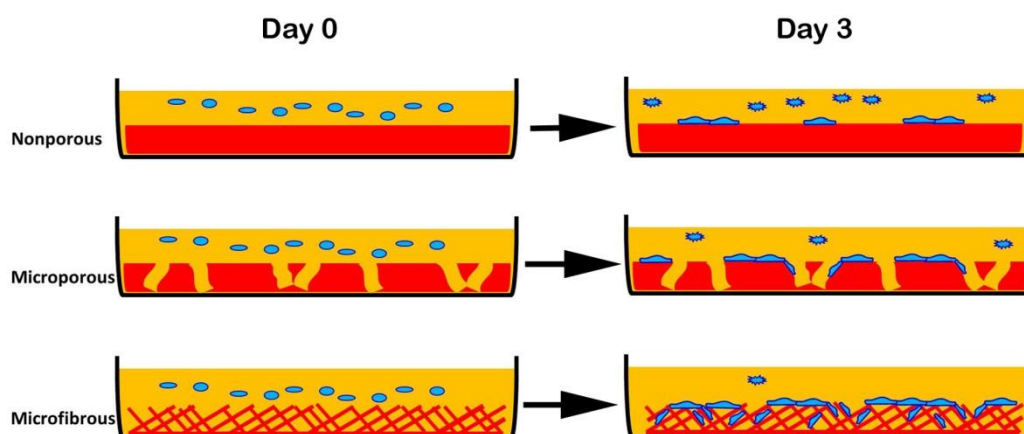


Figure 39: Diagrammatic representation of mGS cell growth on nonporous, microporous, and microfibrillar PCL scaffolds for 3 days. Note that mGS cells preferentially attach and grow on microfibrillar PCL scaffold. Initially (day 0), equal number of mGS cells were seeded on the three scaffolds. By day 3, there are more cells attached on microfibrillar scaffold than those on nonporous or microporous scaffolds. However, the number of floating (unattached) cells in the culture media of nonporous scaffold is more than those on microporous or microfibrillar scaffolds.

support better mGS cell growth than nonporous and microporous scaffolds. This observation is also demonstrated when MTT assay is used to analyze the total live cells attached on the scaffolds and suspended in the media. The DNA PicoGreen assay was also used to estimate the amount of cells floating in the culture media and confirmed the advantage of using microfibrous scaffolds as compared to the two other types. This is also demonstrated when toluidine blue staining and SEM were used. Therefore, scaffold architecture affects mGS cell binding and growth. This is clearly depicted through the diagrammatic representations (Fig. 39).

It is known that cells interact with the extracellular matrix via integrin binding and sense difference in mechanical stresses through integrin signaling. It was shown that increasing porosity is associated with increasing the expression of integrins (Knudson & Loeser, 2002). 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 as compared to nonporous and microporous scaffolds (Figs. 5, 11c, 12c).

A nonporous PCL scaffold provided surface roughness which allowed adhesion and moderate proliferation of cells (Biazar et al., 2011). Microporous scaffolds prepared with the salt-leaching method led to the formation of pores that appeared to moderately facilitate growth and integration of cells on their surfaces (Tessmar et al., 2005). Microfibrous scaffolds fabricated by electrospinning technique appeared to be most suitable for growth of mGS cells for several reasons.

i) The scaffolds acquired micro-size pores with interconnectivity that aids the communication between mGS cells during their growth and proliferation. **ii)** The microfibers acquired surface roughness due to evaporation of solvent during their deposition with high surface area under the effect of high voltage (Biazar et al.,

2011). This surface roughness is expected to enhance cell adhesion. **iii)** The microfibrinous scaffold offered a 3D construct with a larger surface area than that of nonporous or microporous scaffolds due to the interlocking between the non-woven microfibers leading to various shapes and sizes of interconnected pores. **iv)** The microfibrinous scaffold showed a closer similarity in mechanical performance, when subjected to tensile forces, to those of natural stomach tissues. **v)** This similarity could be attributed to the morphological appearance of microfibers of the scaffold which resemble the fibers of extracellular matrix in the connective tissue of the stomach wall (Madurantakam et al., 2009). In this study, the average diameter of the fibers fabricated in the microfibrinous scaffolds is within the normal range of collagen type 1 fibers seen in the extracellular matrix.

Preferential growth of mGS cells on microfibrinous scaffold is not surprising. Recently, it was found that the fibrous architecture of synthetic polymer scaffolds allows stem cells to develop a self-contained microenvironment that supports their proliferation, self-renewal, and even differentiation in combination with soluble cues (Carlson et al., 2012). The authors predicted that their findings would make it possible for stem cells to bypass the need for incorporation of matrix proteins or feeder cells. Studies already showed that the porous topography of the PCL scaffold is self-sufficient to improve cells specialized functions (Liu et al., 2014; Li et al., 2014; McHugh et al., 2014).

This study highlights the value of 3D culture system and the limitations of the 2D cell culture in stem cell research. The pattern of cell growth and cellular biological processes and responses in conventional 2D culture are different from those of animal models. The 3D culture models allow studies onto biological processes in a setting that resembles *in vivo* environments and thus provides more

physiological context. In contrast to matrix and spheroid technologies, the 3D culture models somehow mimic extracellular matrix.

4.4 Establishment of a three dimensional culture model of mGS cells directing their growth and differentiation into gland mucous cells

Since mGS cells preferentially attach and grow on microfibrinous scaffolds after 3 day culture, it was of interest to follow their growth pattern on the same type of scaffold for different time points. Seeding mGS cells on microfibrinous scaffolds for 3-12 days and analyzing their growth pattern made it possible to identify their phenotypic change and the influence of PCL microfibers on cell growth and differentiation program.

The increased DNA content (proliferation) of mGS cells from 3 to 6 days of culture on PCL scaffolds was followed by a significant reduction of the amount of DNA by day 9 suggesting a decrease in cell proliferation rate (Fig. 16). This down-regulation of cell proliferation could be explained by the lack of integrin binding sites on the scaffold. It has been noted that basement membrane plays a critical role in stem cell proliferation and differentiation due to presence of laminin and its binding to integrins. The integrin affect cell proliferation by signalling events mediated through their cytoplasmic domains (Mainiero et al., 1997). Integrin's extracellular domain is also involved in adhesion through interactions with laminin (Simon-Assmann et al., 1995). Targeted deletion of the cytoplasmic domain of integrin induced reduction in cell proliferation and cell cycle arrest (Fang et al., 1996; Zhu et al., 1996). PCL microfibers are inert material lacking the integrin binding sites or laminin that may cause the modification in the cell cycle signalling and directing the stem cell fate.

The reduction in cell proliferation was associated with an increase in the size of 9 day-cultured mGS cells (Fig. 16) which could suggest differentiation of the mGS cells with loss of some of these differentiated or end cells. Increase in cell size can be attributed to the more specialized structure and function. To further clarify this observation, cryosections of mGS cells cultured for 3 and 9 days were processed for lectin- and immunocytochemical probing. At 3 day-culture, mGS cells did not react with any of the examined gastric epithelial cell lineage-specific biomarkers. However, the situation was different for mGS cells cultured for 9 days. Of the various lectins that are known to bind different gastric epithelial cells, GSII showed reactivity with some of the cultured mGS cells (Fig. 21). It is known that GSII binds to N-acetyl-D-glucosamine of mucous granules in the gland mucous cells of the oxyntic/pyloric regions of the mouse stomach (Karam et al., 2004). Furthermore, when antibodies specific for TFF1, TFF2 and alpha/beta subunits of H,K-ATPase (respectively specific for pit, neck and parietal cells) were used for immunofluorescence probing, only anti-TFF2 antibodies reacted with some of the mGS cells cultured for 9 days (Fig. 20b). Also mGS cells grown on coverslips or chamber slides did not bind to any of the biomarkers examined. Since both GSII lectin and anti-TFF2 antibody are known markers of glandular mucous cells, it appears that the mGS cells have differentiated into the gland mucus-secreting cells.

Real time PCR conducted on mGS cells cultured in 3D system for 3-12 days showed changes in the expression pattern of mRNA profiles specific for different genes of stem cell proliferation and differentiation. The stem cell marker Oct4 was up-regulated after 3 days of culture suggesting an enhancement in the pluripotency of the cells and their capability of differentiation. This finding is not surprising. Even though Oct4 is considered as a stem cell marker and the expression is expected to be

down-regulated during differentiation, there are evidences demonstrating that this is not always the case. In migrating primitive endodermal cells, the transient up-regulation of Oct4 expression suggests that Oct4 down-regulation is not required for differentiation (Ovitt & Schöler, 1998). It has also been reported that the ES cells differentiation into neuronal and cardiac cell lineages is associated with increase in Oct4 expression (Shimozaki et al., 2003; Zeineddine et al., 2006). Both Oct4 and LIF pathways have crucial roles in the self-renewal and pluripotency of stem cells. During stem cell differentiation, the down-regulation of LIF gene leads to a decrease in the expression of some target genes underlying pluripotency. But in contrast, Oct4 mRNA and protein remain at high levels for few days (Zeineddine et al., 2014).

Results of both immunocytochemistry and lectin cytochemistry demonstrated the binding of two very well characterized biomarkers: anti-TFF2 antibody (Karam et al., 2004) and GSII lectin (Falk et al., 1994; Karam et al., 2005). It is also known that gastric stem cell differentiation into a glandular mucous cell involves an increase in cell size due to development of the machinery necessary for production of secretory granules (Karam & Leblond, 1993c). Indeed in this study, not only SEM revealed an increased cell size (Fig. 17b), but confocal microscopy also showed the development of GSII-positive secretory granules characteristic of mucous cells (Figs. 21b,c). All these findings together provided a strong evidence for the differentiation of mGS cells into glandular mucous cells.

4.5 Molecular mechanism underlying differentiation of mGS cells into mucous cells

Little is known about the molecular mechanisms involved in the differentiation of gastric mucus-secreting cells. In the corpus region of the mouse

stomach, the stem cells gradually develop into pre-neck cell progenitors which are characterized by a slight development of the Golgi apparatus and formation of prosecretory granules at its trans face. Further development of the Golgi apparatus and formation of a few small cored secretory granules defines the pre-neck cells. These two steps (preneck cell progenitor and preneck cell) are not associated with an increase in cell size (Karam & Leblond, 1993c).

Mature mucus-secreting neck cells are characterized by a well-developed Golgi apparatus producing numerous large cored secretory granules. These granules are packed in throughout the cytoplasm and lead to the enlargement of the cell. The neck cells are not end cells. After about 2 weeks of going through several cycles of mucus synthesis and secretion, the mucous neck cells start to change their phenotype by producing secretory granules containing an increasing amount of pepsinogen at the expense of mucus. Therefore, gradually mucous neck cells transform into prezymogenic cells which eventually become zymogenic cells (Karam & Leblond 1993c).

The transcription factor MIST1 was identified as a regulator for the differentiation of mucous neck cells into zymogenic cells (Ramsey et al., 2007). In addition, the transcription factor XBP1 is required for turning off the progenitor features of neck cells and the induction of MIST1 needed for the development of zymogenic cells (Huh et al., 2010). Recently, in mice, XBP1 was also found to be involved in the development of the mammary glands and differentiation of their epithelial cells (Hasegawa et al., 2015). In the mouse intestine, XBP1 was also found to regulate the crypt base columnar stem cells (Niederreiter et al., 2013).

In the present study, the expression of XBP1 in mGS cells is demonstrated (Fig. 19b). Moreover, with the growth of mGS cells on microfibrinous scaffolds for 3-

12 days, there is a significant decrease in the expression of XBP1, which correlates with the differentiation into gland mucus-secreting cells. Therefore, it seems that XBP1 is not only important for the terminal differentiation of mucous cells into zymogenic cells, but also for the early development of mucous cells from the stem cells and their immediate descendants.

XBP1 is the downstream target gene of androgen receptor which is influenced by Forkhead box A1 (FOXA1). Studies in endometrical cancer cells showed that XBP1 transcription needs both androgen receptor and FOXA1 expression. These 2 factors together are also required for the activation of Notch signaling (Qiu et al., 2014). Therefore, in our 3D culture system of mGS cells, down-regulation of XBP1 suggests the inhibition of Notch signaling possibly due to down-regulation in the upstream target, androgen receptor. Notch inhibition activates several genes including SPDEF.

In the mouse stomach (antrum and corpus regions), the transcription factor SPDEF is expressed in mucus-secreting gland/neck cells and is required for terminal differentiation of antral gland mucous cells (Horst et al., 2010; Noah et al., 2010). In the intestinal epithelium, SPDEF was also found to be expressed in the mucus-secreting goblet cells. In addition, it was expressed in Paneth cells as well as the crypt base stem/progenitor cells (Gregorieff et al., 2009; Noah et al., 2010). Knockout of SPDEF in the intestine was associated with down-regulation of the differentiation and production of both goblet cells and Paneth cells (Gregorieff et al., 2009). Interestingly, induction of SPDEF expression in colon cancer LS174T cell line was associated with their differentiation into mucus-secreting goblet cells (Noah et al., 2010). In the present study, the expression of SPDEF in mGS cells and its gradual up-regulation with their growth on microfibrous scaffolds was demonstrated

(Fig. 19a). These findings together with the XBP1 down-regulation and immuno/lectin cytochemical data (TFF2 localization and GSII binding) provide an explanation for the differentiation into gland mucous cells.

The up-regulation of SPDEF expression is associated with enhancement of other genes including AGR2 which is also known to be expressed in mucous neck cells (Chen et al., 2009; Gupta et al., 2013). SPDEF expression blocks the proliferation of progenitor cells.

In the present study, down-regulation of PCNA supports this suggestion. Down-regulation of XBP1 and lack of the expression of MIST1 suggests that mucous neck cells did not proceed into further levels of differentiation. It is also reported previously that AGR2 expression in mucous cells in the stomach promotes differentiation of multiple cell lineages, while inhibiting the proliferation of stem cells. Loss of AGR2 leads to the depletion of parietal cells and chief cells and hyper-proliferation of mucous neck cells (Gupta et al., 2013).

In the present study, up-regulation of DCLK1 suggests a role for notch signaling in the differentiation of mGS cells (Qu et al., 2014). Studies published in 2011 suggest that DCLK1 may be a posttranscriptional regulator of *miR-144* micro RNA downstream targets such as Notch 1. DCLK1 inhibition leads to the reduction of HES1 and increase in the expression of *miR-144* indicate its regulation of notch signaling (Sureban et al., 2011a,b).

This *in vitro* model will help to study the effect of many pharmacological agents against SPEM (Spasmodic polypeptide expressing metaplasia) as it seems to resemble the same cell type formation. This 3D culture system will hopefully help in defining the molecular mechanisms involved in the differentiation of gastric stem cells to mucus-secreting cells as well as other gastric cell lineages. This 3D model

will help in answering the questions of DCLK1 role in regulating differentiation through notch inhibition as well as over expression. The role of AGR2 and Oct4 in the carcinogenesis also can be studied using this system as the SPDEF is a known enhancer of AGR2 (Karam, 2012; Obacz et al., 2015).

The octamer-binding transcription factor 4 (Oct4) belongs to the POU family of proteins and binds octamer DNA motifs in the promoters of several genes to regulate the pluripotency of stem cells (Pan et al., 2002). An increased expression of Oct4 causes differentiation of embryonic stem cells into primitive endoderm and mesoderm. Down-regulation of Oct4 induces dedifferentiation and formation of trophoctoderm (Niwa et al., 2000). In human adipose tissue stem cells, over-expression of Oct4 and Sox2 enhances proliferation and induces differentiation into adipocytes and osteoblasts (Han et al., 2014). In embryonic stem cells, the Oct4 associates with recombining binding protein suppressor of hairless (RBPJ), a transcription factor that acts as the nuclear effector of the Notch signaling pathway (Bray, 2006; Lake et al., 2014; Li et al., 2012; van den Berg et al., 2010) suggesting the involvement of Oct4 in Notch signaling pathway. Oct4 also inhibits the FOXD3-dependent activation of the FOXA1 and FOXA2 endodermal promoters in embryonic cells (Guo et al., 2002). FOXA1 and androgen receptor are involved with the Notch signaling regulation (Qiu et al., 2014).

Collectively, the results of this study indicate that microfibrous PCL scaffolds support growth of mGS cells and trigger their differentiation into mucus-secreting glandular cells. Gene expression analysis indicates that multiple regulatory genes are involved in this differentiation program (Fig.40).

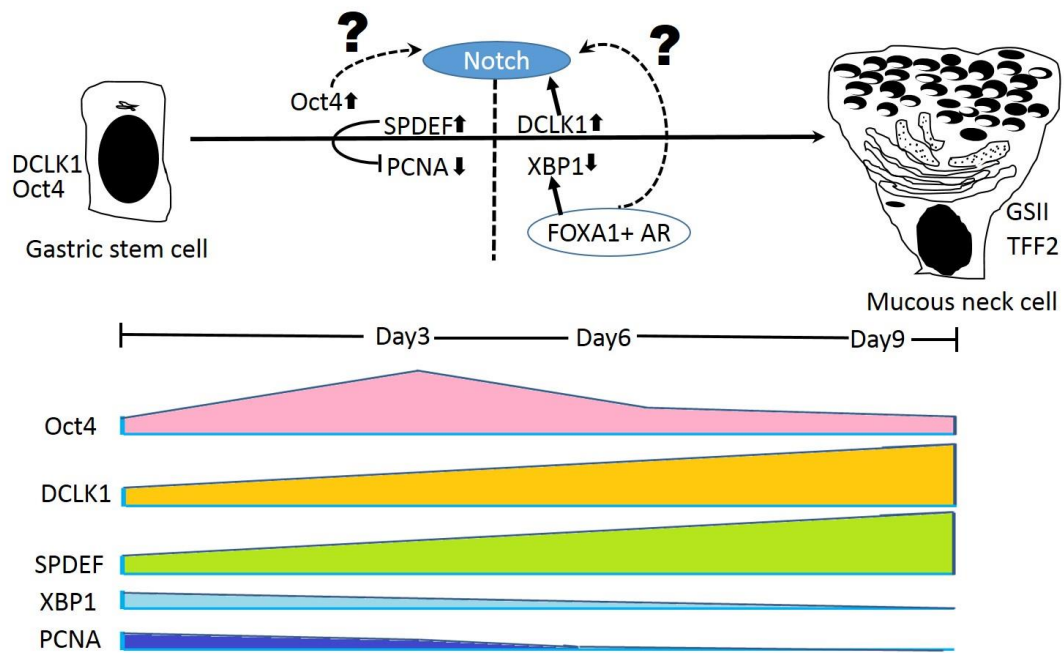


Figure 40: Diagram representing the differentiation of gastric stem cell into gland mucous cell after 9-day-culture on microfibrinous PCL scaffold and the changes that occur in the expression pattern of genes involved. The mGS cell expresses DCLK1 and Oct4 and mucous neck cell is GSII and TFF2 positive. This differentiation process is associated with down-regulation of XBP1 and PCNA and increase in the mRNA levels of DCLK1 and SPDEF. Note that Oct4 is initially up-regulated (day 3) and then down-regulated by days 6 and 9. This differentiation process involves Notch signaling via Oct4, SPDEF, DCLK1, or even FOXA1 and AR (androgen receptor).

4.6 Features of mGS cells and PCL scaffolds in acidic environment

The extracellular environment plays a significant role in cell proliferation and differentiation (Heylings et al., 1984). Several studies have shown that changing the extracellular pH has different effects on the cellular functions. The acidic pH affects 1) the growth properties of chinese hamster embryonic fibroblast cell lines (Ober & Pardee, 1987), 2) the cellular metabolism and protein synthesis in bone marrow stromal cell (Kohn et al., 2002), 3) the hematopoietic cells with the activation of lymphocytes, neutrophils and proliferation of macrophages and production of erythropoietin, 4) the rate of erythroid cell differentiation (McAdams et al., 1997, 1998), 5) the phosphorylation of Akt and MAPKs in human esophageal microvascular endothelial cells and inducing Hsp27 and Hsp70 in human esophageal microvascular endothelial cells (Mauchley et al., 2010; Rafiee et al., 2006), and 6) the survival, migration, proliferation, and differentiation of the oligodendocyte precursor cells (Jagielska et al., 2013).

In case of mGS cells, the present study showed different parameters of their growth and behavior following their seeding on 3D microfibrinous PCL scaffolds using RPMI media at different acidic pH values. In addition, the expression of genes involved in their proliferation, pluripotency, and differentiation was analyzed. Finally, the mechanical properties and chemical nature of the microfibrinous PCL scaffolds were analyzed after exposure to acidic RPMI media at different pH values.

4.6.1 Survival of mGS cells and inhibition of their migration at pH 6.0

When mGS cells are incubated in RPMI media at acidic pH values for 5 hr, their viability is greatly compromised. The acidic pH values (3 or 4) of RPMI culture media leads to a significant reduction in the viability of mGS cells and induction of

their death. It seems that the acidic media induced breaks in the cell membrane allowing the uptake of propidium iodide indicating cell death. But at pH of 4.5 there is an uptake of both calcein and propidium iodide suggesting that the cells are going through a transitional stage where they are still retaining some mitochondrial enzymatic activity but at the same time have started to deteriorate and develop pores on their membranes allowing some incorporation of the propidium iodide.

At pH 5, most of mGS cells incorporated calcein and there was a low level of cell death. However, the attachment of the cells was highly affected and they detached in small sheets. When the growth and viability of mGS cells on the 3D microfibrinous PCL scaffold was tested in a mild acidic environment (pH 5.5), there was no significant difference when compared to cells growing in normal conditions (pH 7.4). Therefore, the growth and viability of mGS cells in RPMI at pH 5.5 did not change with changing the culture condition from 2D to 3D.

When the migration behavior of mGS cells was tested in the acidic environment by using the wound healing assay, there was a significant inhibitory effect on cell migration. By comparing the width of the wound in mGS cells cultured at pH 7.4 with those at pH 6.0, there is more than 5-fold difference in the width of the wound after 2 days of its induction suggesting a significant inhibition in the migration of mGS cells in the acidic environment (Fig. 25).

4.6.2 Microfibrinous PCL scaffolds sustain harsh acidic environment

To test whether the 3D culture system established in this study could be useful for future gastric tissue engineering experiments with regeneration and transplantation applications, it is mandatory to know whether the transplantable

scaffold is able to sustain the pH condition of the stomach until the transplanted epithelium integrates with the surrounding tissues.

The maximum stress achieved by scaffolds incubated in acidic environment is compared to that of mouse stomach and the scaffold incubated in pH 7.4 media. It was very clear from this study that, the effect of incubation at pH 5.5 for up to 12 days is minimal and the peak stress obtained showed insignificant change. Even though pH 3.0 affects the peak stress produced, it is still above the peak stress obtained for that of mouse stomach. This effect could be due to some changes in hydrophobicity or loss of connections between fibers resulting in loosening of the meshwork organization of the microfibers. Despite this slight decrease in the mechanical integrity of the scaffold treated at low pH values, there were no signs of chemical change in the polymer after extensive investigation using IR spectroscopy (Fig. 28).

4.6.3 Enhanced expression of mucous cell-specific genes in 3D culture of mGS cells at pH 5.5

The 2D and 3D culture conditions have different impact on the behavior of mGS cells. In the present study, the differentiation of mGS cells on 3D culture is demonstrated. Testing whether the acidic pH (5.5) has any effects on the molecular markers checked in 2D and 3D at pH 5.5 and 7.4 revealed significant differences in their profile.

The stem cell marker DCLK1 mRNA expression showed a significant pattern in both 2D and 3D culture conditions. In 2D culture, at pH 5.5, by day 3 the mRNA expression of DCLK1 is increased by 12 folds and, interestingly, down-regulated by day 9. In 3D culture, at pH 5.5, the scenario is different; by day 3, there is a 2-fold

increase in DCLK1 expression which is then down-regulated by day 9. The function of the DCLK1 protein is broad. Its tubulin binding domain is involved in shaping the cytoskeleton, thereby regulating cell motility, cell cycle as well as differentiation. The protein kinase function and the presence of several phosphorylation sites suggest its involvement in signaling pathways (Sossey-Alaoui & Srivastava, 1999). Also, DCLK11 expression is confirmed at the later stage of differentiation of enterocytes (Bjerknes et al., 2012). Notch signaling induction is needed for differentiation of enterocytes and its inhibition will be associated with down-regulation of DCLK1 positive stem cells and enterocytes and enhancement in the production of mucus-secreting goblet cells and hormone-secreting enteroendocrine cells (Milano et al., 2004; Qu et al., 2014).

In the present study, the up-regulation of DCLK11 may indicate that the stemness is reduced in 3D culture when compared to 2D system and the acidic pH significantly up-regulated the mRNA expression of DCLK11 indicating the cells are driven into the mode of differentiation. The proliferation marker, PCNA showed significant down-regulation in both 2D and 3D culture conditions. However, it is up-regulated on day 3 at pH 5.5. PCNA expression had correlation with DCLK11 expression where the stemness is reduced the proliferation is also reduced confirming that the cells are going to the stage of differentiation.

This possible enhancement of the differentiation of mGS cells in the acidic environment was confirmed when the expression of SPDEF gene was examined. The mGS cells were analyzed after 3-day culture on microfibrous PCL scaffolds using RPMI at pH 5.5. Using primers specific for SPDEF and qRT-PCR revealed a remarkable increase in its mRNA expression level.

4.6.4 Precocious differentiation of mGS cells into mucous cells

In the present study, microfibrinous PCL scaffold is found to support mGS cell growth and differentiation into gland mucous cells after 9-day culture. Biomarkers specific for different gastric epithelial cells did not bind to cryosections of mGS cells growing on PCL scaffolds for 3 days in normal pH. Whereas on day 9, the cells bound to GSII lectin confirming their differentiation into gland mucous cells. At acidic pH (5.5), mGS cells grown on microfibrinous scaffolds for 3 days showed positive staining to GSII lectin indicating that low pH enhanced the differentiation process. While changing the pH in the media greatly changed the mRNA expression in both 2D as well as 3D culture systems and clearly gives the impact that pH is an important factor driving the cells to differentiate. Gastric stem cells are located in the isthmus region of the gastric gland near the luminal surface. Previous studies showed that the acid-secreting parietal cells are the key component of gastric stem cell niche influencing their growth and differentiation (Bredemeyer et al., 2009). This also points into the fact that the acidic environment has an influential role in regulation of gastric gland homeostasis.

The gastric acid plays a major role in the pathogenesis of the gastro-esophageal reflux disease and associated abnormalities in the differentiation program of the epithelium leading to the precancerous metaplastic changes. Clinical studies showed that low pH exposure induces alternation in the differentiation program of stem cells in the lower esophagus (Chiu et al., 2009). In addition, acid exposure study on human esophageal epithelial cells is associated with the production of ATP, interleukins and up-regulation of mRNA and protein expression for the acid-sensing transient receptor potential cation channel indicating the role of acidic environment

in regulating gene expression and inducing injury (Ma et al., 2012; Rafiee et al., 2009). In another study, chronic acid exposure to esophageal epithelial cells induced CDX2 expression in long term culture suggesting transdifferentiation into an intestinal like epithelium (Marchetti et al., 2003). Similarly chronic acid exposure induced colonic phenotype in the non-neoplastic Barrett epithelial cell line (Bajpai et al., 2008). In the intestine, the acid output from the stomach is also implicated in the metaplastic changes that happen in the lining epithelium. When the intestinal epithelial cells were incubated with acidified media at pH 5 to 6.5 for 3 days, it was associated with down-regulation of CDX2 and sucrose isomaltase and up-regulation of gastric mucins MUC5ac and MUC6 (Faller et al., 2004). Studies using immortalized human colon carcinoma cell line HT29 showed that long term exposure to pH 5.0 leads to cell differentiation whereas the short pulse exposure leads to enhanced cell proliferation mediated by Na/H exchanger (Fitzgerald et al., 1997). Similarly, 3-min acid exposure at pH 6.0 enhanced cell proliferation in Barrett's esophageal adenocarcinoma cells (Sarosi et al., 2005).

The findings of the present study and the effects of acidic pH on the proliferation and differentiation program of mGS cells could provide an explanation for the clinical scenario of chronic atrophic gastritis which is associated with loss of parietal cells and change of the pH in the gastric lumen leading to amplification of gastric epithelial progenitor/stem cells associated with up-regulation of Oct4 and eventually cancer development (Al-Awadhi et al., 2011; Al-Marzoqee et al., 2012).

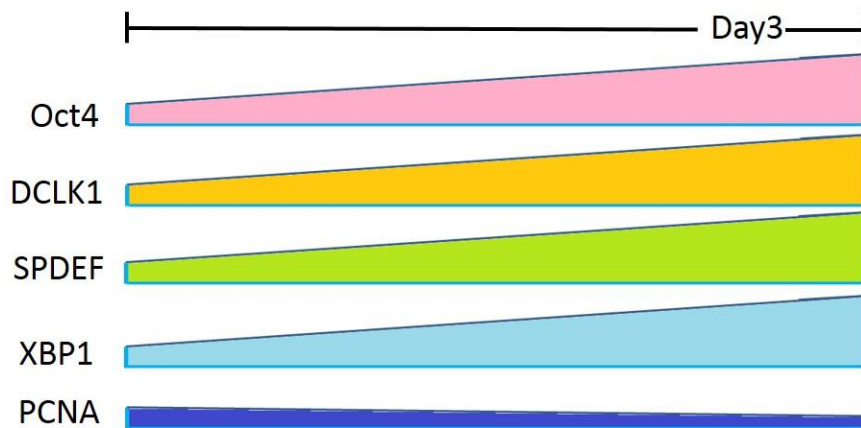
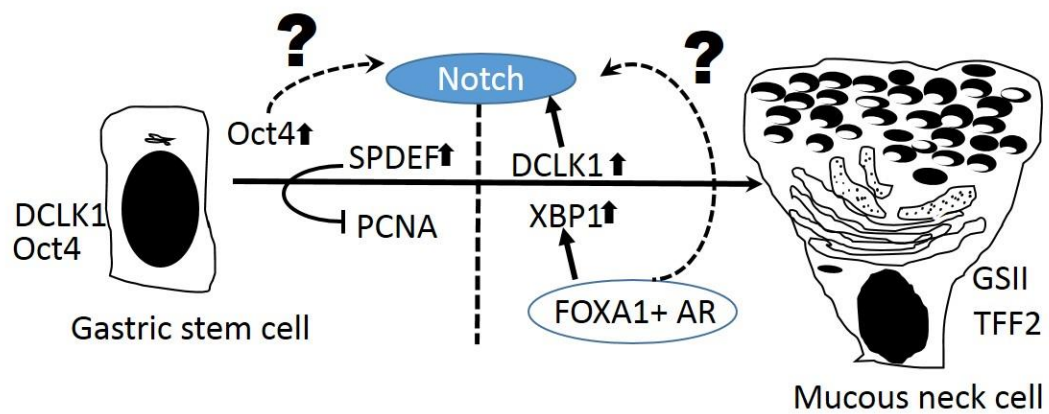


Figure 41: Diagram representing the differentiation of gastric stem cell into gland mucous cell after 9-day-culture in acidic pH on microfibrinous PCL scaffold and the changes that occur in the expression pattern of genes involved. The mGS cell expresses DCLK1 and Oct4 and mucous neck cell is GSII and TFF2 positive. This differentiation process is associated with down-regulation of PCNA and increase in the mRNA levels of XBP1, DCLK1, and SPDEF. Note that Oct4 is initially up-regulated (day 3) and then down-regulated by day 9. This differentiation process involves Notch signaling via Oct4, SPDEF, DCLK1, or even FOXA1 and AR (androgen receptor).

Chapter 5: Conclusion

This study explores the possible use of gastric epithelial stem cells in tissue engineering for applications in regenerative therapy. Establishment of a 3D culture model of mGS cells demonstrates their differentiation into mucus-secreting neck cells similar to those in the gastric glands. Since the long-term plan of this research is to make use of this culture model for *in vivo* studies and transplantation application, and since the mGS cells will become exposed to acidic pH of the stomach, it is mandatory to know how the viability and regenerative potential of mGS cells will be affected before extending this study to any animal application. Previous studies showed that the pH is an important factor that affects growth and differentiation of esophageal and intestinal epithelial cells. Surprisingly, little is known about the effects of gastric acid secretion on the stem cells of the stomach itself. The present study, highlight the importance of the role of acidic pH in controlling stem cell proliferation and differentiation. In the 3D culture system established in the present study, the mGS cells are able to tolerate acid pH down to 5.5 without affecting their viability and adherence. It is also demonstrated that this acidic environment enhances mGS cell differentiation and speeds up the development of mucous neck cells and formation of GSII-positive mucous granules.

The possible future development and use of this 3D culture system for transplantation experiments will require some pre-requisites. The intraluminal pH of the stomach should be controlled perhaps by using a proton pump inhibitor or an H2 receptor antagonist to ensure that the pH will not reach below 5.5 and to ensure maximum survival, proliferation, and speedy differentiation of the transplanted stem cells. However, it is not known how the existing mature cells of the stomach such as

those secreting pepsinogen will affect the transplanted mGS cells. Therefore, some co-culture experiments will be needed to evaluate how the mGS cells will behave in the presence of neighboring mature gastric epithelial cells and also the underlying mesenchymal cells.

In addition of being useful in setting up the basis for gastric tissue engineering for regenerative treatment of some stomach diseases, this newly established 3D culture model of gastric stem cells will help in elucidating and dissecting the signaling pathways involved in the process of gastric stem cell differentiation such as Notch signaling. In addition, this culture system will make it possible to test the effects of different pharmaceutical agents, new synthetic/natural compounds, and growth factors or hormones on gastric stem cell proliferation and differentiation. These numerous values of the 3D mGS cell culture model will be of much benefit to many clinicians and scientists in the field of gastroenterology.

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