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Role of EGF mediated laminin γ2 chain in uterine cervix carcinoma

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Abstract

Carcinogenesis is a multistep transformation process of normal cells to a neoplastic state. The microenvironment that surrounds cancer cells also act on malignant transformation as a functional network. Uterine cervix carcinoma is the fourth most common malignancy in women worldwide, being diagnosed annually 528 000 new cases and 266 000 related-deaths were observed in 2012. In Portugal, the incidence and mortality rate are relatively high, being the incidence 10.8 and mortality 4.9 cases per 100,000 persons per year. There are several histological types of uterine cervix cancer, however the two most common are squamous cell carcinomas and adenocarcinomas, comprising 75–90 and 10–25 % of all cases, respectively.

In last years, it has been given more importance to tumor microenvironment as an important factor in carcinogenesis and disease progression. Extracellular matrix (ECM) is a major component of microenvironment, being composed by a complex network of glycoproteins such as collagens, laminins, fibronectins and proteoglycans. Laminins are a group of large extracellular glycoproteins and a major constituent of the basement membrane (BM) compartment of ECM. Laminin-332 is a specific subtype of laminins in the BM, having a trimeric structure composed by α3, β3 and γ2 chains. High levels of laminin-332 expression were found in several human cancers, being considered a poor prognosis factor and have been related to the invasive ability of several tumors, such as uterine cervix cancer. Furthermore, the cytoplasmic accumulation of γ2 chain (LAMC2) has been implicated in uterine carcinoma progression and has been frequently found at the invasive front of tumors, being associated with a poor survival, recurrence, and metastasis. The proteolytic processing of LAMC2 chain affects the dynamics of cellular adhesion and expose the EGF-like repeats of this molecule. EGF is considered the main growth factor acting on uterine cervix cancer.

This work aims to clarify the EGF mediated role of LAMC2 of laminin-332 in the progression of uterine cervix carcinomas, using *in vitro* models of squamous cell carcinoma (SiHa) and adenocarcinoma (HeLa). In order to achieve our aim, we defined five specific aims to verify: (i) the role of EGF in cell cycle and proliferation; (ii) the effect of EGF stimulation in the expression of LAMA3, LAMB3, LAMC2, LAMC1 and LAMC3; (iii) the role of LAMC2 in EGF effect in cell cycle, proliferation and migration/invasion; (iv) the effect of LAMC2 knockdown in the expression of LAMC1 and LAMC3, and (v) the transcription factors involved in EGF dependent regulation of LAMC2 and LAMC1 expression.

The analyses of human cancer databases confirmed that LAMC2 is upregulated in several cancer types and its expression is also increased in uterine cervix cancer. The cell cycle analyses revealed that both cell lines are EGF-responsive, so we confirmed that EGF is a suitable growth factor to stimulate uterine cervix cancer cells. Our results showed that EGF stimulation results in a shortened duration of G0/G1 cell cycle phase and in an increased percentage of cells in S+G2/M phases, concomitant with increased cyclin D1 levels. It was also found that EGF regulates the expression of LAMC2 in both cell lines (squamous cell carcinoma and adenocarcinoma). Both FOXM1 (in SiHa) and STAT3 (in HeLa) seems to be crucial for LAMC2 regulation.

To investigate the role of LAMC2 in uterine cervix carcinoma, it was performed a *LAMC2* silencing through shRNA technology. LAMC2 knockdown showed that EGF stimulates proliferation independently of LAMC2 in SiHa cells, but interestingly in HeLa the pro-proliferative effect of EGF is more efficient in the absence of LAMC2. Moreover, the LAMC2 silencing suppresses SiHa ability to migrate and invade. The matrix metalloproteinases (MMPs) are the most relevant family of proteinases involved in extracellular matrix turnover, acting as tumor microenvironment modulators. The activity of MMP2 and 9 is regulated by EGF and the levels of activity of MMP9 is related to LAMC2 levels, in both cell lines. In SiHa, the MMP2 activity is most affected by LAMC2 knockdown, which results in a

decrease of MMP2 activity. So, it seems that the malignant phenotype of squamous cell carcinoma relies more on LAMC2 than the malignant phenotype of adenocarcinoma.

LAMC1 association with cancer is controversial, however, the LAMC2 knockdown induces the transcription of *LAMC1* in SiHa at mRNA level. Under control conditions, the same result was observed for *LAMC3*, although it had a lower significance which disappeared with EGF treatment. In wild type (WT) cell lines upon EGF stimulus the protein levels of LAMC1 increases in HeLa not in SiHa. It seems that this laminin gene can play a role in an EGF rich microenvironment, conferring a mild malignant phenotype in adenocarcinoma (low invasive profile). Again, EGF also plays a role in LAMC1 expression in SiHa and HeLa cells and these regulation seems to be associated to FOXM1 and STAT3, though the real meaning of this action must be unraveled.

We believe this thesis gives relevant insights on the role of regulatory dynamics of LAMC2 by EGF that accounts for the uterine cervix squamous cell carcinoma aggressive phenotype; and it also pointed LAMC1 as a putative key element in uterine cervix cancer progression.

Keywords: uterine cervix cancer, tumor microenvironment, epidermal growth factor (EGF) laminin γ2 (LAMC2), cancer cell proliferation, migration/invasion

Resumo

A carcinogénese é um processo complexo e gradual de transformação pelo qual as células normais originam células com potencial neoplásico. Durante este processo, as células cancerígenas adquirem características de malignidade, que resultam da acumulação de várias mutações genéticas e alterações epigenéticas, levando assim, à ativação de oncogenes e inativação de genes supressores de tumores. Essas caraterísticas são designadas de *hallmarks* do cancro, sendo que as principais são: autossuficiência em relação aos sinais de crescimento, insensibilidade aos sinais de anti-crescimento, resistência à apoptose, elevado potencial replicativo, aumento do potencial angiogénico, evasão ao controlo imunitário, reprogramação metabólica e capacidade de invasão tecidular e metastização. O microambiente que envolve células cancerígenas também atua neste processo como uma rede funcional, que inclui células normais, fatores mediadores e componentes da matriz extracelular.

O carcinoma do colo útero é o quarto tipo de cancro mais comum em mulheres em todo o mundo, sendo diagnosticados anualmente 528 000 novos casos. Em 2012 ocorreram 266 000 mortes relacionadas com este tipo carcinoma. Em Portugal, a taxa de incidência e mortalidade é relativamente alta, sendo a incidência de 10,8 e a mortalidade de 4,9 (taxas por 100.000 indivíduos por ano). Existem vários tipos histológicos de cancro do colo do útero, no entanto, os dois tipos mais comuns são os adenocarcinomas e os carcinomas pavimento-celulares, que compreendem 75-90 e 10-25% de todos os casos, respetivamente.

O vírus do papiloma humano (HPV- *human papillomavirus*) é transmitido sexualmente e está bem estabelecido o seu papel como agente etiológico de vários tipos de cancro na área anogenital, incluindo o cancro de colo do útero. Aproximadamente 70% dos cancros do colo do útero estão diretamente associados aos sorotipos HPV16 e HPV18. Os genes E6 e E7 dos HPV de alto risco são oncogenes que desregulam o controlo do ciclo celular, podendo originar a transformação maligna das células.

A matriz extracelular (ECM – *extracellular matrix*) é um componente chave do microambiente tumoral, sendo que nos últimos anos foi reconhecido o seu papel no processo carcinogénico. A ECM é composta por uma rede complexa de macromoléculas como colagénios, lamininas, fibronectinas e proteoglicanos. A sua principal função é o suporte tecidular, no entanto, também participa no controlo de eventos celulares como proliferação celular, adesão, migração, invasão e apoptose.

As lamininas são glicoproteínas extracelulares de elevado peso molecular, sendo um dos principais componentes estruturais dos filamentos de ancoragem presentes nas membranas basais (BM - *basement membrane*). Todas as lamininas são proteínas heterotriméricas, que contêm três cadeias, denominadas α, β e γ. Atualmente estão descritas 16 lamininas com diferentes propriedades que resultam da conjugação das diferentes subunidades. A sua expressão é altamente regulada durante o desenvolvimento e a sua distribuição é tecido-específica. A laminina-332 (anteriormente denominada laminina 5) é um subtipo de laminina específica das BM dos epitélios, sendo a sua forma trimérica constituída pelas cadeias α3, β3 e γ2. Nos tecidos normais, a laminina-332 interage com as integrinas α3β1, α6β1 e α6β4. Esta interceção é muito importante na adesão celular e por essa razão também está envolvida na migração e a invasão celular.

Atualmente, a importância da laminina-332 é um assunto de destaque em diversas revisões bibliográficas, onde foram descritas novas funções estruturais e reguladoras desta macromolécula em vários carcinomas. Os tumores epiteliais (carcinomas) muitas vezes secretam grandes quantidades de laminina-332 e frequentemente expressam o seu ligando, a integrina α6β4. O aumento da expressão da laminina-332 tem sido observado em vários tipos de cancro, tendo sido considerado um fator de mau prognóstico. Foi também relacionado com a capacidade invasiva de vários tumores, como o carcinoma do colo do útero. De facto, sabe-se que a laminina-332 pode ativar vias de sinalização, uma vez que, níveis elevados desta proteína podem estimular a ativação da proteína cinase ativada por mitogénio (MAPK -*mitogen activated protein kinases*) e da fosfatidilinositol 3-cinase (PI3K -*phosphatidylinositide*

3-kinase), levando assim a um aumento da capacidade proliferativa e invasiva das células e à estimulação do crescimento tumoral.

A laminina γ2 humana (LAMC2) é produto da expressão do gene *LAMC2* e, como as outras cadeias, possui duas variantes transcricionais, resultantes do *splicing* alternativo do exão terminal 3'. No entanto, esta é a cadeia que confere especificidade ao trímero da laminin-332. Vários estudos mostraram o aumento da expressão da LAMC2 em cancros humanos, incluindo adenocarcinomas de estômago, pâncreas, tiroide, língua, colo-retal, pulmão, colo do útero, esófago, cabeça e pescoço, pele e pulmão. Observou-se que a acumulação citoplasmática da LAMC2 ocorre frequentemente na frente invasiva dos tumores e está associada a uma diminuição da sobrevivência dos doentes, à recidiva e formação de metástases. Relativamente ao cancro do colo do útero, alguns estudos mostraram que existe uma elevada expressão da cadeia LAMC2 em lesões microinvasivas e invasivas. Apesar da expressão da cadeia LAMC2 ter sido descrita em adenocarcinoma e em carcinomas pavimento-celulares do colo do útero, a sua acumulação ocorre predominantemente nos carcinomas pavimento-celulares.

A formação e remodelação da ECM são ativamente reguladas por proteólise, que contribui para a homeostase dos tecidos. Contudo, no contexto tumoral, podem ocorrer desequilíbrios na proteólise, levando assim, a um crescimento tumoral desregulado, remodelação do tecido, inflamação, invasão e metastização. As MMPs (*matrix metalloproteinases*) são a família de protéases mais relevante associada à carcinogénese. Estas enzimas estão descritas como moduladoras do microambiente tumoral, uma vez que tem um papel crucial na regulação da matriz extracelular e na regulação de vias de sinalização relevantes para a progressão tumoral. As gelatinases MMP2 e MMP9 são as MMPs mais proeminentemente envolvidas na degradação da BM e, consequentemente, estão envolvidas no desenvolvimento tumoral e na formação de metástases. A hidrólise da cadeia γ2 por MMPs parece estar relacionada com o aumento da migração e invasão celular em carcinomas, uma vez que o processamento da cadeia LAMC2 afeta a dinâmica da adesão celular. A hidrólise da cadeia LAMC2 tem sido associada à atividade da MMP2 em cancros da mama e do colo do útero.

O fator de crescimento epidérmico (EGF-*epidermal growth factor*) compreende onze polipéptidos, que compartilham um domínio EGF conservado, sendo esta uma das famílias de fatores de crescimento mais relevantes na progressão tumoral. Os vários ligandos podem ser produzidos quer por células cancerígenas quer por células do estroma, e a sua ligação a recetores de cinase de tirosina, conhecidos como recetores do EGF (EGFRs), estimulam vias de sinalização intracelulares específicas. A ativação dos recetores cinase tirosina culminam na ativação as cascatas enzimáticas envolvidas no crescimento e na sobrevivência das células, sendo que as vias MAPK e PI3K são as mais ativadas. Em cancro, a maioria das mutações que afetam o EGFR são mutações *nonsense* que promovem a sua ativação constitutiva, tornando a sua ativação independente da presença do ligando-EGF. Contudo, os recetores EGFR e HER2 estão frequentemente sobre-expressos, podendo funcionar como oncogenes. No cancro do colo do útero, o EGFR parece ser o recetor do EGF mais relevante. O domínio III da laminina γ2 apresenta segmentos EGF-*like*, que podem interagir com os recetores de EGF, nomeadamente o EGFR, levado assim à ativação das cinases da via das MAPK.

O principal objetivo deste trabalho foi esclarecer o papel da LAMC2 de laminina-332 na progressão mediada por EGF de carcinomas do colo uterino, utilizando modelos *in vitro* de carcinoma pavimento-celular (SiHa) e adenocarcinoma (HeLa). Para atingir este objetivo foram delineados 5 objetivos específicos no sentido de verificar: i) o efeito do EGF no ciclo celular e na proliferação; (ii) a relevância da estimulação pelo EGF na expressão da LAMA3, LAMB3, LAMC2, LAMC1 e da LAMC3; (iii) a importância da LAMC2 no efeito do EGF no ciclo celular, proliferação e migração/invasão; (iv) o efeito do *knockdown* da LAMC2 na expressão da LAMC1 e da LAMC3, e (v) os fatores de transcrição envolvidos na regulação da expressão da LAMC2 e da LAMC1, dependente de EGF.

Para a primeira abordagem, foram usadas bases de dados com amostras de doentes, que confirmaram, como já tinha sido descrito por outros autores, que a LAMC2 é sobre-expressa em vários tipos de cancro, incluindo o cancro do colo do útero. De forma a validar o efeito do EGF nos modelos *in vitro*, foram feitos ensaios de ciclo celular. Estes resultados mostraram que ambas as linhas celulares são responsivas ao EGF, uma vez que proliferam mais após o estímulo. Além disso, os nossos resultados mostraram ainda que a estimulação com EGF resulta num encurtamento da duração da primeira fase do ciclo celular, G0/G1, levando ao aumento do número de células nas fases S+G2/M. Este facto é acompanhado pelo aumento concomitante dos níveis da ciclina D1. Verificou-se ainda, que o EGF também regula a expressão de LAMC2 nas duas linhas celulares (SiHa-carcinoma pavimento-celular e HeLa-adenocarcinoma). Os ensaios de imunoprecipitação da cromatina, deram-nos bons indícios de que o FOXM1 (em SiHa) e o STAT3 (em HeLa) são fundamentais na regulação de LAMC2 pelo EGF.

De forma a compreender melhor o papel da LAMC2 no cancro do colo do útero, a expressão da *LAMC2* foi silenciada com shRNA. Os ensaios de proliferação após o *knockdown* da LAMC2 mostraram que o EGF estimula a proliferação independentemente da LAMC2 nas células SiHa, mas curiosamente nas células HeLa, o efeito proliferativo do EGF é mais eficiente na ausência de LAMC2. Além disso, o silenciamento LAMC2 suprime a capacidade migratória e invasiva das células SiHa, mas não parece afetar a migração das células HeLa. Relativamente à atividade das MMPs, ambas MMP2 e 9 são reguladas pelo EGF e os níveis de atividade de MMP9 estão relacionados com os níveis de LAMC2, em ambas as linhas celulares. A atividade da MMP2 também é afetada pelos níveis da LAMC2, contudo, é afetada de forma oposta comparando ambas as linhas celulares. Nas células SiHa, o *knockdown* da LAMC2 diminui a atividade de MMP2, enquanto que nas células HeLa a atividade desta enzima é aumentada. Assim, parece que o fenótipo maligno dos carcinomas pavimento-celulares é mais depende da expressão da LAMC2 do que os adenocarcinomas.

Além disso, o *knockdown* da LAMC2 resultou no aumento da transcrição da *LAMC1* nas células SiHa. Em condições controlo, o mesmo resultado foi observado para a LAMC3, contudo, este efeito desaparecia após o estímulo com EGF. Portanto, devia às alterações mais significativas ocorrerem na regulação da LAMC1 esta foi a cadeia que apresentou mais interesse ao nível da carcinogénese. No entanto, nas linhas celulares *wild type (WT)* sob a influência de EGF os níveis da LAMC1 apenas aumentaram nas células HeLa. Isto sugere que esta cadeia pode desempenhar um papel no fenótipo maligno moderado em adenocarcinoma (perfil de baixa invasão) em microambientes ricos em EGF. Novamente, o EGF também desempenha um papel na expressão de *LAMC1* em ambas as linhas celulares, que parece estar associado aos fatores de transcrição FOXM1 e STAT3. O real significado desta regulação há que ser estudado mais em pormenor. De forma a tentar entender o papel da LAMC1 em contexto tumoral analisámos as mesmas bases de dados. Observámos, contudo, que a associação entre a expressão de LAMC1 e cancro do colo do útero é muito controversa.

Assim, esta tese fornece informações relevantes sobre o papel da dinâmica regulatória da expressão da cadeia LAMC2 pelo EGF, podendo explicar o fenótipo mais agressivo do carcinoma pavimento-celular do cancro do colo do útero. Além disso, também sugere que a expressão da LAMC1 pode ser um elemento chave na progressão do cancro do colo do útero. Portanto, novas perspetivas de investigação podem ser delineadas a partir dos nossos resultados, de forma a compreendermos melhor os mecanismos moleculares subjacentes às várias formas de progressão inerentes aos diferentes tipos histológicos do cancro.

Palavras-chave: cancro do colo do útero, microambiente tumoral, fator de crescimento epidérmico (EGF), laminina γ2 (LAMC2), proliferação e migração/invasão tumoral

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List of abbreviations, acronyms and symbols

AA – Antibiotic-antimycotic solution
AP-1 – Activator protein 1
ATCC – American Type Culture Collection
BLCA – Bladder urothelial carcinoma
BM – Basement membrane
BRCA – Breast invasive carcinoma
BSA – Bovine serum albumine
CDK – Cyclin-dependent kinase
cDNA – Complementary DNA
CESC – Cervical squamous cell carcinoma and endocervical adenocarcinoma
ChIP – Chromatin immunoprecipitation
CHOL – Cholangiocarcinoma
CO_2 – Carbon dioxide
COAD – Colon adenocarcinoma
COADREAD – Colon/rectal adenocarcinoma
ddH_2O – Sterile ultra-pure water
DMEM – Dulbecco's modified Eagle media
DNA – Deoxyribonucleic acid
dNTPs – Deoxynucleotides
DT – Population doubling time
DTT – Dithiothreitol
ECM – Extracellular matrix
EDTA – Ethylenediamine tetraacetic acid
EGF – Epidermal growth factor
EGFR – Epidermal growth factor receptor
ERK – Extracellular signal–regulated kinases
ESCA – Esophageal carcinoma
FBS – Fetal bovine serum
FGF – Fibroblast growth factor
FOXM1 – Forkhead box protein M1
G0 – Cell cycle "resting phase"
G1 – Gap 1 cell cycle phase
G2 – Gap 2 cell cycle phase
GEO – Gene Expression Omnibus
HER-2 – Human epidermal growth factor receptor 2
HGF – Hepatocyte growth factor
HNSC – Head and neck squamous cell carcinoma
HPRT – Hypoxanthine-guanine phosphoribosyltransferase
HPV – Human papillomavirus
kDa – Kilodalton
KIRP – Kidney renal papillary cell carcinoma
LAMA3 – Human laminin α3 chain

LAMB3 – Human laminin β3 chain
LAMC1 – Human laminin γ1 chain
LAMC2 – Human laminin γ2 chain
LAMC3 – Human laminin γ3 chain
LUAD – Lung adenocarcinoma
LUSC – Lung squamous cell carcinoma
M – Mitosis cell cycle phase
MAPK – Mitogen-activated protein kinase
miR-29 – microRNA 29
MMP – Matrix metalloproteinase
mRNA – Messenger RNA
NF-kB – Factor nuclear kappa B
PAAD – Pancreatic adenocarcinoma
PAGE – Polyacrylamide gel electrophoresis
PBS – Phosphate buffered saline
PCPG – Pheochromocytoma and paraganglioma
PI – Propidium iodide
PI3K – Phosphatidylinositol 3-kinase
PRAD – Prostate adenocarcinoma
qRT-PCR – Quantitative real time polymerase chain reaction
READ – Rectum adenocarcinoma
RNA – Ribonucleic acid
RNA-Seq – RNA sequencing
RSEM – RNA-Seq by Expectation Maximization
S – Synthesis cell cycle phase
shRNA – Short hairpin RNA
SKCM – Skin cutaneous melanoma
STAD – Stomach adenocarcinoma
STAT3 – Signal transducer and activator of transcription 3
STES – Stomach and esophageal carcinoma
TCGA – The Cancer Genome Atlas
TGF-β – Transforming growth factor-β
TGS – Tris-glycine-sodium dodecyl sulfate
THCA – Thyroid carcinoma
TNF-α – Tumor necrosis factor-α
UCEC – Uterine corpus endometrial carcinoma
ZEB1 – Zinc finger E-box binding homeobox 1

1. Introduction

1.1 Cancer biology

Cancer is a multistep transformation process of normal cells to a neoplastic state, termed carcinogenesis. During this process cancer cells acquire features of malignancy, resulting from the accumulation of several alterations that allow them to become tumorigenic and ultimately malignant. Therefore, carcinogenesis needs genetic mutations and epigenetic alterations that lead to the activation of oncogenes (growth promoting), with dominant gain of function and/or increased expression, and into the inactivation of tumor suppressor genes (growth inhibitory) with recessive loss of function and/or decreased expression [1]. In 2000, Hanahan and Weinberg, defined six cancer features so-called hallmarks of cancer, which are: sustaining proliferative signaling, evasion to growth suppressors, cell death resistance, replicative immortality, angiogenesis capacity, and increase invasive and metastasis ability [2]. The microenvironment that surrounds cancer cells also act on malignant transformation as a functional network, where intervenient cells (malignant and normal) can share chemokines and energy molecules [3].

1.2 Uterine cervix cancer

Uterine cervix cancer is the fourth most common cancer in women worldwide, being diagnosed 528 000 new cases annually and 266 000 related-deaths were observed in 2012 [4]. In Europe, uterine cervix carcinoma is the fifth most common cancer among women. Concerning Portugal, this is the sixth most common and deadly cancer among women. Its incidence and mortality are higher than in the other Southern European countries, being respectively estimated in 10.8 and 4.9 (rates per 100,000 person per year). Regarding prevalence, this cancer is the fourth most prevalent in Portugal, affecting most patients (approximately 52.2%) in a period of five years or more [5]. There are several histological types of uterine cervix cancer, however the two most common are squamous cell carcinomas and adenocarcinomas, comprising 75–90 and 10–25 % of all cases, respectively [6].

Human papillomavirus (HPV) is sexually transmitted and it was demonstrated as an etiological agent of several cancers in anogenital area, including uterine cervix cancer [7]. It has been firmly established the biological and epidemiological casual role of HPV in all uterine cervix cancer cases [8], wherein 70% of these cancers are directly caused by 16 and 18 HPV types [9,10]. E6 and E7 genes of high risk HPVs are oncogenes that deregulate key cell cycle controls, being able to origin the malignant transformation of cells [11].

1.3 Extracellular matrix and cancer (ECM)

Extracellular matrix (ECM) is a relevant component of the tumor microenvironment. In the last years, it has been recognized as an important intervenient in the carcinogenic process. ECM is composed by a complex network of macromolecules, such as collagens, laminins, fibronectins and proteoglycans, giving to this structure distinctive physical and biochemical properties. In homeostasis, its main function is tissue support, however, it also participates in the control of cellular events such as cell proliferation, adhesion, migration, invasion and apoptosis [12].

The ECM deregulation benefits cancer progression, since during carcinogenesis, the remodeling of microenvironment leads to an increased release of ECM-associated growth factors, which will act on cancer and normal cells. In addition, it is also known that tumor cells modulate ECM to facilitate communications and escape the homeostatic control [12,13].

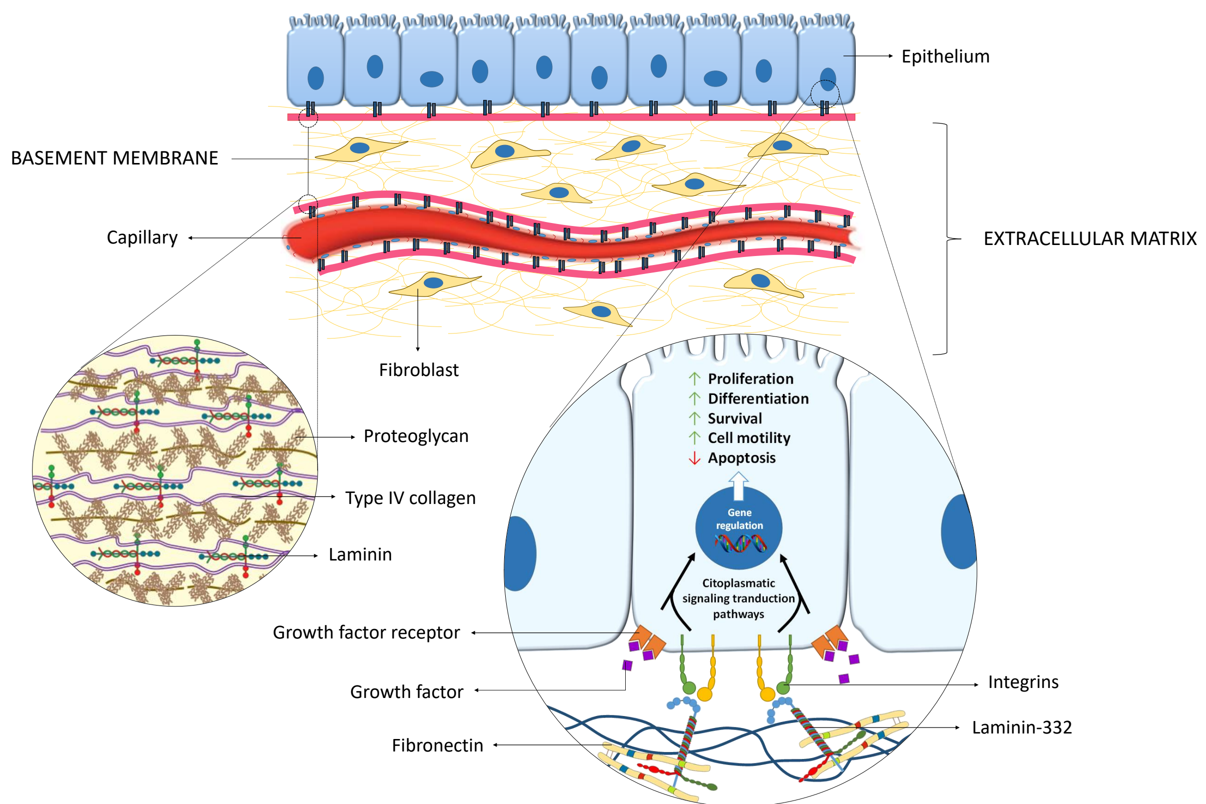


Figure 1.1 – Main components and organization of extracellular matrix (ECM). ECM is composed by a complex structure of macromolecules, such as collagens, laminins, fibronectins and proteoglycans, giving to this network distinctive physical and biochemical properties. Besides tissue support, ECM also participates in the control of cellular events such as cell proliferation, differentiation, adhesion, migration, invasion and apoptosis. ECM molecules regulate the availability of growth factors and cytokines and they also interact with integrins and growth factor receptors. This way ECM can activate specific cytoplasmic signaling transduction pathways, which in turn, regulate the expression of genes involved in these cellular events (adapted from [14]).

1.3.1 Laminin-332

Laminins are large extracellular glycoproteins that are a structural component and a major constituent of the anchoring filaments in the hemidesmosomal complex within the basement membrane (BM) compartment of ECM. All laminins are heterotrimeric proteins that contain three chains, termed α, β and γ. At present, five α, three β and three γ chains are described [15]. There are 16 known laminins with different properties resulting from the conjugation of different subunits. Their expression is highly regulated during development and these proteins have a tissue specific distribution [15–18]. It is known that both epithelial and mesenchymal cells contribute to the deposition of laminin into the basement membrane [19].

Laminin-332 (formerly termed laminin 5) is an epithelial-BM specific subtype of laminin and it is a trimer composed by α3, β3 and γ2 chains. This isoform is present in the BM of the skin and other organs, contributing for the maintenance of epithelial-mesenchymal cohesion [20]. In normal tissues, laminin-332 interacts with integrins α3β1, α6β1 and α6β4 , promoting cell adhesion, migration and invasion, accounting for disease spreading [18,21].

Human laminin-332 precursor molecule is a high-molecular weight of 460 kDa, which upon secretion, originates 440 kDa and 400 kDa isoforms, resulting from extracellular processing of the α3 and γ2-chains [20]. The coiled-coil structure is formed by domains I and II of each of the three chains. Domain III of laminin-332 γ2 chain is an EGF-like domain, which can interact with EGFR. The globular domain is composed by five repeating segments at the structure base. The first three repeats are EGF-

-like sequences (G1-3 domains) that have binding sites for integrin and growth factors receptors. The last two repeats contain a heparin/proteoglycan binding activity [22].

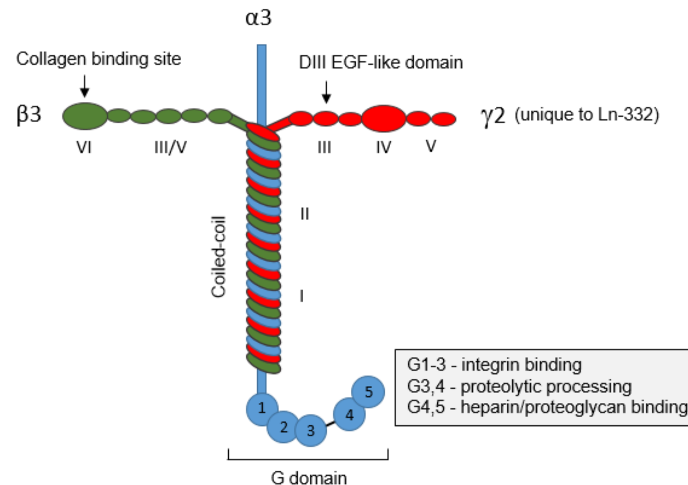


Figure 1.2 – Schematic illustration of laminin-332 structure: laminin-332 forms a cross-shaped structure containing three chains (α3, β3 and γ2). The main-specific binding sites for each chain are identified (adapted from [23]).

1.3.2 Laminin-332 and cancer

The importance of laminin-332 is a highlight subject in diverse reviews where it was described new structural and regulatory functions of this macromolecule in several carcinomas. It has been suggested that this protein is implicated in carcinoma progression and analysis of protein expression has been used as an invasion diagnostic marker and prognostic tool [17,23].

Despite controversial studies, the abnormal expression of laminin and its integrin receptors is used as a mark of certain tumor types. Epithelial tumors (carcinomas) often secrete abundant amounts of laminin-332 and frequently express its ligand α6ß4integrin [24]. High levels of laminin-332 expression were found in several human cancers by immunohistochemical studies, being considered a poor prognosis factor and have been related to the invasive ability of several tumors, such as uterine cervix carcinoma, pancreatic carcinoma, hypopharyngeal cancer, urothelial cancer, small-sized lung adenocarcinoma, malignant glioma, gastric cancer, squamous cell carcinoma of the tongue, colorectal adenoma and hepatocellular carcinoma [18].

Laminin-332 has been identified as an activator of signaling pathways in cell. The high levels of large globular domain 4-5 of α3 chain expression was already demonstrated in carcinoma, *in vivo*, where it stimulates the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) pathways. This alteration results in an increase of invasive capacity of cells and tumor growth, which could be reverted using blocking-antibodies against this domain of α3 chain [25]. Regarding β3 and γ2 chains, there are findings relating the cleavage of these chains by specific proteases with the increase in cell migration and invasion in carcinomas. The domain III of laminin γ2 fragment is EGF like, being able to bind to EGFR and leading to the activation of extracellular signal–regulated kinases 1/2 (ERK1/2) from MAPK pathway [23].

1.3.3 Laminin γ2

Human laminin γ2 chain (LAMC2) is the product of the *LAMC2* gene expression and like the other chains it has two transcript variants, resulting from alternative splicing of the 3' terminal exon. The predicted molecular weight of long isoform is 131 kDa while for the short isoform is 122 kDa. However, it has the distinction of being specific to the laminin-332 trimer. Moreover, it is the only chain of the trimer that can be secreted as a monomer, remaining unclear its biological relevance [23].

Several immunohistochemistry and *in situ* hybridization studies showed strong expression of LAMC2 in many human cancers, including adenocarcinomas of the stomach [26], colon [27], pancreas [28], thyroid [29], tongue [30], colorectal [31], lung [32], uterine cervix [33], and squamous carcinomas of the esophagus [34], head and neck [35], skin [36], lung [37], as well as transitional cell carcinoma of the bladder [38]. The cytoplasmic accumulation of LAMC2 has been frequently found at the invasive front of tumors, being associated with a poor survival, recurrence, and metastasis [22].

The processing of LAMC2 chain affects the dynamics of cellular adhesion. Moreover, LAMC2 chain undergoes proteolytic cleavage by specific enzymes as matrix metalloproteinases (MMPs) (MT1-MMP and MMP2) [39]. The proteolysis of LAMC2 occur in the short arm of the molecule, resulting in a 100- or 105-kDa subunits, which harbors EGF-like repeats, allowing it to interact with cell surface receptors [23], as EGFR, on which depends the activation of cell migration, invasion and proliferation[40]. This growth factor-like role of LAMC2 fragments are sustained by the increased levels of both EGFR and LAMC2 [23] in cancer cells, creating a positive-feedback loop in which EGF-EGFR binding can enhance the expression of LAMC2 [22].

Regarding uterine cervix cancer, some studies have shown elevated LAMC2 chain expression in microinvasive and invasive lesions [33,41]. Despite the expression of LAMC2 chain has been described in both squamous cell carcinomas and adenocarcinoma, in uterine cervix it is more frequently accumulated in squamous cell carcinoma [42].

1.4 Extracellular matrix proteolysis: Matrix metalloproteinases

ECM formation and remodeling is regulated by proteolysis and contribute to tissue homeostasis. However, in cancer context proteolysis imbalances lead to deregulated tumor growth, tissue remodeling, inflammation, invasion and metastasis. The MMPs are the most relevant family of proteinases associated with tumorigenesis. Because of their role in extracellular matrix turnover and in the regulation of signaling pathways that are relevant for cancer progression, MMPs have been described as tumor microenvironment modulators [43].

MMPs are a family of zinc-dependent endopeptidases. In the cancer process, besides tumor cells, stromal, endothelial and inflammatory cells are capable of secreting MMPs. Those enzymes are capable of cleaving several macromolecules of the ECM, including laminins [43,44].

Gelatinases MMP2 and MMP9 are the prominent MMPs involved in basement membrane degradation and, consequently, involved in cancer development and metastasis [44]. Cleavage of LAMC2 chain have been associated to MMP2 activity in breast [45] and in uterine cervix cancers [46].

1.5 Growth factors and cancer

Growth factors are a class of compact polypeptides with the capacity to bind to transmembrane receptors harboring kinase activity domains that are localized at the cytoplasmic region of the molecule, stimulating specific intracellular signaling pathways [47]. The kinase proteins have the ability to phosphorylate specific amino acid residues, such as serine, threonine and tyrosine. The tyrosine kinase receptors phosphorylate tyrosine amino acid residues of several intracellular protein, which activates enzymatic cascades involved in cell growth and survival, however, MAPK and PI3K pathways are the mostly activated ones [48]. Those growth factors assume an important role in tumor initiation, by stimulation cell survival and clonal expansion, which permits fixation of oncogenic mutations, as well as tumor progression, invasion, angiogenesis and metastasis [48]. Epidermal growth factor (EGF), which comprises eleven polypeptides sharing a conserved EGF domain, is a relevant growth factor family in tumor progression [48,49].

The EGF ligands can be produced by cancer and stromal cells and it binds to tyrosine kinase receptors known as EGF receptors (EGFRs) [49]. Each receptor comprises an extracellular domain to allow ligand

binding, a single transmembrane portion, and an intracellular protein tyrosine kinase domain [47]. In human cancers the EGFR and HER2 receptors are frequently overexpressed, which can result in the activation of proto-oncogene transcription factor [50,51]. EGFR is frequently affected by non-sense mutations that promote its constitutive activation, which releases it from the need of EGF stimuli to act [52]. Alterations of these receptors were already described in non-small cell lung, bladder, uterine cervix, ovarian, kidney, pancreatic and head and neck cancer [48]. However, in uterine cervix cancer EGFR is so far the most relevant EGF receptor [51,53].

1.6 Regulation of *LAMC2* expression

The ECM-mediated and cytokine-mediated signaling pathways are intrinsically linked and this cross--talk can influence the composition of the basement membrane [54,55]. In intestinal epithelial cells, both transforming growth factor-β1 (TGF-β1) and hepatocyte growth factor (HGF) cytokines stimulate LAMC2 expression, result of activator protein-1 (AP-1) DNA binding sites activation on LAMC2 promoter region [54]. Besides that, it was also demonstrated that tumor necrosis factor-α (TNF-α) regulates LAMC2 transcription by an nuclear kappa B (NF-κB) bound enhancer [56]. In colon and pancreatic cancer cells, LAMC2 is under positive transcriptional control of Smad4, which in turn, is increased by TGF-β [57].

In vitro studies showed that the enhanced expression of LAMC2 is regulated by Wnt/β-catenin signaling pathway in gastric and colorectal cancers [31,58]. The frizzled receptor activation after Wnt binding results in an increase of β-catenin that induces indirectly the transcription of LAMC2 due to transcript factors binding to the AP-1 binding sites of LAMC2 promoter [22,58]. Furthermore, unlike the others laminin-332 chains, the *LAMC2* gene expression is activated by zinc finger E-box binding homeobox 1 (ZEB1) transcript factor in colorectal cancers [59].

There are studies that associated the decreased expression of LAMC2 in lung, prostrate, breast and basal cell skin carcinoma to aberrant methylation of the LAMC2 promoter region [22]. On the other side, in gastric cancer, the LAMC2 is frequently overexpressed, being this pattern associated with promoter demethylation and histone modifications [60].

Moreover, recent studies have shown the significant downregulation of miR-29a/b/c in several cancers including uterine cervix cancer [61,62]. These microRNAs molecules modulate the expression of their target genes post-transcriptionally blocking mRNA translation. LAMC2 mRNA was identified as target of miR-29s [61]. Thus, the decreasing of miR29 expression levels in cancer cells might result in an upregulation of LAMC2 expression [22]. Altogether, this regulation pathways lead to a significant increase of LAMC2 release into extracellular compartment and consequently heterotrimerization followed by deposition in BM.

2. Aim of the project

This work aims to clarify the EGF mediated role of LAMC2 of laminin-332 in the progression of uterine cervix carcinomas, using an *in vitro* model of squamous cell carcinoma and adenocarcinoma.

In order to achieve our aim, we defined five specific aims to verify: (i) the role of EGF in cell cycle and proliferation; (ii) the effect of EGF stimulation in the expression of LAMA3, LAMB3, LAMC2, LAMC1 and LAMC3; (iii) the role of LAMC2 in EGF effect in cell cycle, proliferation and migration/invasion; (iv) the effect of LAMC2 knockdown in the expression of LAMC1 and LAMC3, and (v) the transcription factors involved in EGF dependent regulation of LAMC2 and LAMC1 expression.

3. Materials and methods

3.1 Cell lines and culture conditions

SiHa, a human uterine cervix squamous cell carcinoma (HTB-35, ATCC) and HeLa, a human uterine cervix adenocarcinoma (CCL-2, ATCC) cell lines were used as *in vitro* models of uterine cervix cancer. SiHa cells (-squamous cell carcinoma) are positive for HPV16 and HeLa (adenocarcinoma) are positive for HPV18 [63]. They were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle media (DMEM 1x) (41965-039, Gibco, Life Technologies) in a humidified atmosphere of 5% CO_2 at 37 °C. The culture medium was supplemented with 10% fetal bovine serum (FBS) (S 0615, Merck) and 1% of antibiotic-antimycotic solution (AA) (P06-07300, PAN Biotech). Cells were grown to 75 - 100% optical confluence before they were detached with 0.05% trypsin-EDTA 1X. To determine the cell number necessary for each assay a Neubauer counting chamber was used.

For gene expression analysis by qRT-PCR (3.6), cells (2.5×10^5 cells/mL) were plated in 6-well plates and then exposed to growth factor (EGF- E9644, Sigma-Aldrich, MO, USA). After cells became adherent, they were synchronized under starvation (culture medium without FBS) for 8h. Cells were then cultured in normal (control) medium or exposed to 25ng/mL of EGF. Culture supernatants were analyzed by zymography (3.9).

For cell cycle analysis and proliferation curves (3.3 and 3.4), cells were plated in 48-well, when adherent they were submitted to starvation (~8h) and collected for analysis at 0, 6, 12, 24, 30 and 48h after EGF (25ng/mL) supplementation. Control curves were defined in the same time points using cells cultured in the absence of EGF.

For immunofluorescense (3.5), cells were grown on Millicell® EZ SLIDE, until they reached approximately 80% of confluence, or were attached to a glass slide by cytospin method (1200 rpm for 5 min) (Shandon CytoSpin II Cytocentrifuge). Cells were submitted to starvation and EGF stimulated as described above.

For wound healing assay, cells (2.5×10^5 cells/mL) were plated in 12-well and cultured until reaching a confluent monolayer. To inhibit cell proliferation, that could mask migration results, they were treated with Mitomycin-C (5 µg/mL) (M4287, Sigma), an antiproliferative agent, for 3 h prior starting the experiment. The effect of EGF was tested by exposing cells in the described above conditions and comparing with cells cultured in the absence of EGF, then cells were analyzed as presented in 3.7.

3.2 Bioinformatics analysis

LAMC2 expression was analyzed in several tumor groups, using data from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO). The RNA-Seq data (RSEM) were extracted. All disease groups with normal and tumor samples were considered to the present analysis.

3.3 Flow cytometry for cell cycle analysis

In order to divide, cells need to grow and replicate their DNA in a process known as cell cycle. The four major phases of the cell cycle are Gap 1 (G1), Synthesis (S), Gap 2 (G2), and Mitosis (M). The G1 phase precedes the DNA replication that occurs in S phase, and after G2 phase the cells finally divide in M phase[64]. G0 is the quiescent phase in which cells are not dividing. Using DNA staining dyes such as propidium iodide (PI) (P4170, Sigma-Aldrich), the DNA content in the cells at G0/G1, S, and G2/M phases can be accurately quantified. Technically the method/protocol we used by flow cytometry do not allow to distinguish G0 phase from G1 phase and G2 phase from M phase [65].

After exposure to EGF as described in 3.1, cells were harvested and fixed in 70% ethanol at 4 ºC for at least 16 h. Cells were then centrifuged at 1200 rpm for 5 min, the supernatant was discarded and cells

were incubated with 100 μL of PI solution (50μg/mL PI, 0.1mg/mL RNase A, 0.05% Triton X-100) for 40min at 37 ºC. After washing with PBS 1X, cells were centrifuged 1500 rpm 10min 4 ºC and the supernatant was discarded. Cells were then suspended in 200 μL of PBS-BSA 0.1%. The flow cytometry analysis was performed in a FACScalibur (Becton Dickinson). Data were analyzed with FlowJo software, after excluding death cells and cell aggregates the univariate model was applied.

3.4 Cell proliferation curves and calculation of population doubling time (DT)

The cell number was calculated using a Neubauer chamber and viable cells were determined using 0.4% (w/v) trypan blue stain at a ratio of 1:4 or using flow cytometer counter. Population doubling time (DT) is the time required for a culture to double the number of cells and it was calculated according to ATCC® Animal cell culture Guide, using the following formula:

$$3.1\ \mathrm{DT} = \frac{\ln 2}{\ln\left(\mathrm{Xe}/\mathrm{Xb}\right)}\ \mathrm{x}\ \mathrm{T}$$

where T is the incubation time in any units, Xb is the cell number at the beginning of the incubation time and Xe is the cell number at the end of the incubation time.

The duration of a particular phase of the cell cycle can be predicted using the following formula [66]:

$$3.2\ \mathrm{Tx} = \frac{ln\ (\mathrm{FS}+1)}{\ln 2}\ \mathrm{x}\ \mathrm{DT}$$

where Tx is the duration of cell cycle phase of interest (e.g. G0/G1 phases, S phase, G2/M phases), DT is the duration of cell cycle and FS is the fraction of cells in the cell cycle phase of interest, estimated from the DNA content frequent histogram.

3.5 Immunofluorescence

Immunofluorescence is a technique used to identify specific biomolecules in a cell, using specific antibodies labeled with fluorochromes, directly or indirectly, allowing their visualization and localization in the cell. In this work, indirect immunofluorescence was performed using two antibodies: a primary antibody that binds to the molecule of interest and then a secondary antibody covalently labeled with a fluorophore, which binds to the primary antibody.

Cells were fixed in 2% (w/v) paraformaldehyde (104003, Merck Millipore) for 15 minutes at 4 ºC, blocked with 0.1% (w/v) PBS-BSA for 30 minutes at room temperature, and incubated with primary antibody overnight (diluted in 0.1% (w/v) PBS-BSA, 1:100). Antibodies used were monoclonal anti-LAMA3 (BM165, kindly provided by Dr. Patricia Rousselle, Institut de Biologie et Chimie des Protéines, Lyon, France [67]), polyclonal anti-LAMB3 (PA5-21514; Thermo Scientific, Sweden), monoclonal anti-LAMC2 (MAB19562; Chemicon, Germany), monoclonal anti-LAMC2 (LS-C152903, LifeSpan BioSciences, USA), monoclonal anti-LAMC1 (AMAb91138, Atlas Antibodies, Sweden) . After washes, cells were incubated with the secondary antibodies for 2 hours, at room temperature. Secondary antibodies (diluted in 0.1% (w/v) PBS-BSA, 1:1000) used were: Alexa Fluor® 488 anti--mouse (A-11001, Invitrogen) and Alexa Fluor® 488 anti-rabbit (A-11034, Invitrogen). Negative controls were stained without primary antibodies to ensure the specificity of the secondary antibody. After washing three times with PBS, samples were counterstained with VECTASHIELD media with DAPI (H-1200, Vector Labs, CA, USA). Cells were examined by standard fluorescence microscopy using a Nikon Instruments Eclipse Ti-S Inverted Microscope (Hamamatsu digital camera C10600 ORCA-R2) and an Olympus IX53 Inverted Microscope dedicated to fluorescence. Images (x200 field) were acquired and processed with NIS-Elements AR-3.2.software and Olympus cellSens software, respectively, and quantified with ImageJ software.

3.6 RNA extraction, reverse transcription and relative quantifying real-time polymerase chain reaction (qRT-PCR)

Real-time PCR is an accurate and sensitive technique that combines both amplification and detection in one step. Gene transcription activity can be evaluated by mRNA quantification, although this analysis can be somehow affected by different mRNA stability and translational rates. The complementary DNA (cDNA) is synthesized from mRNA using a reverse transcriptase. Then cDNA is used as a template in real-time[68]. The qRT-PCR analysis is normalized for a housekeeping gene, which is constitutively expressed in cells[69].

RNA was extracted using RNeasy Mini Kit Qiagen® (74104, Qiagen), according to the manufacturers' protocol. RNA concentration of each sample was measured at 260nm, in a NanoDrop 2000 (ND-2000, Thermo Scientific). The RNA was then converted to cDNA, using 1µg of total RNA, by incubation into a buffer with random primers (11034731001, Roche)) at 70 °C (primers annealing temperature), for 10 min, then, at 4 ºC was added a mixture with: First Strand Buffer 5X (Y00146, Invitrogen), reverse transcriptase (SuperScript™ II) (18080-44, Invitrogen), dithiothreitol (DTT) (Y00147, Invitrogen), RNAse OUT™ (10777-019, Invitrogen), deoxynucleotides (dNTPs) mix (10mM) (28-4065-22V, 28-4065-02V, 28-4065-12V and 28-4065-32V, GE Healthcare), and purified water (ddH_2O) up to 12 µL. Relative quantifying PCR was performed using cDNA, specific pairs of primers for each gene and SYBR® Green Master Mix (04707516001, Roche) according to manufacturer's instructions in Lightcycler® 480 System instrument (05015243001, Roche). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as housekeeping gene. Primer sequences are presented in *Supplementary Table 1*.

3.7 Wound healing assay

Wound healing assay is a method used to measure directional cell migration *in vitro*, mimicking cell migration during wound healing *in vivo* [70]. This assay was performed in order to identify a potential role of EGF, as well as the effect of LAMC2, on cell migration.

In each cell monolayer, a scratch was made to the diameter of the well, and the wound healing was followed by acquiring phase-contrast images (x200 field) at the following time points: 0, 6, 24, 32 and 48 hours.

3.8 Invasion assay

Transwell inserts (8-µm pore size) (PI8P01250, Millicell) were used to determine the effect of LAMC2 knockdown on invasion of uterine cervix cell lines *in vitro*.

For invasion assay, we coated the filter of the transwell inserts with 50 µl of Matrigel. Cells ($1x10^5$ per well) were resuspended in 200 µl FBS-free medium and placed in the upper chamber for each group (shControl or shLAMC2). The lower chamber was filled with 500 µl medium containing 10% FBS as a chemoattractant and incubated for 48 h. At the end of the experiment, cells from the upper chamber were removed using a cotton swab. After wash twice into cold PBS 1x, cells on the lower surface of the insert's filter were fixed using 70% methanol overnight at -20 ºC. Then, cells were stained with 0.5% crystal violet in methanol (25%) for 10 min at room temperature. After washing with 10% methanol, phase-contrast images were taken (x200 field).

3.9 Zymography

Zymography is an electrophoretic technique useful for analyzing the activity of hydrolytic enzymes, such as the matrix metalloproteinases, based on the enzyme specific substrates. In this method, performed with native polyacrylamide gel (PAGE), the proteins are separated according to their hydrodynamic size. MMPs activity is detected based on the degradation of gelatin incorporated in PAGE [71].

Media supernatants of cell line were concentrated by using Amicon® Ultra-4 Centrifugal Filter Units (UFC800308, Millipore). After electrophoresis with TGS buffer 1X (161-0772, Bio-Rad) (150V for 90 minutes) in a 12% PAGE with 0.1% (w/v) gelatin, gel was incubated in renaturating buffer (25% TritonX-100 (v/v)) for 1 hour with agitation, and lastly, was incubated overnight at 37°C, in collagenase buffer (6.06 g Tris-base (T6066, Sigma), 11.7 g sodium chloride (1.06404, EMD Millipore), 0.55 g calcium chloride (1.02382, EMD Millipore) and distilled water up to 100 mL (pH 7.6)). Staining was performed with 0.5% (w/v) Coomassie Blue R-250 (27816, Sigma) for 30 minutes and distaining with distilled water.

3.10 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) is an experimental technique used to investigate the interaction between proteins and chromatin in a cell [72]. In this work it was used to determine whether specific proteins, such as transcription factors, were associated with specific genomic regions. Cells were treated with 37% formaldehyde to crosslink proteins and DNA keeping the chromatin structure, and terminated with 0.125M glycine. After, samples were sonicated and the size of the chromatin fragments was evaluated by electrophoresis gel, in a 1.2% (w/v) agarose, having fragments mainly with a size between 1000bp and 500bp. The chromatin complexes were immunoprecipitated with 1 µL (~2µg/mL) of specific antibodies: rabbitanti-FOXM1 (NBP1-30961, Novus Biologicals, United Kingdom) and rabbit anti-phospho STAT3 (Tyr705) (9145S, Cell Signaling Technology, MA, USA). ChIP assay was performed using OneDay ChIP kit (kch-onedIP-060, Diagenode) according to the manufacturer's protocol. Primers were designed to amplify a putative FOXM1 and STAT3 binding sites at the LAMC1 and LAMC2 promoters. Amplification of promoter regions sequenced from released DNA was performed by qRT-PCR as described in 3.6. The relative occupancy of binding sites was calculated using the following formula:

$$3.3\ \text{Relative occupancy} = 2^{(\text{CtNegCtl} - \text{CtTarget})}$$

3.11 Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0 software (www.graphpad.com). Data for each study parameter from each group were presented as the mean (normal distribution) or median (non-normal distribution) ± SD. Assays were performed with, at least, 3 replicates per condition. Comparisons between data from each group were statistically analyzed by a two-tailed unpaired Student's *t*-test. Differences were considered statistically significant at $p<0.05$.

4. Results

4.1 LAMC2 is increased in cancer *versus* normal tissues

In order to understand the significance of LAMC2 chain in cancer context, *LAMC2* mRNA expression data from normal tissue and tumor were analyzed. The dataset was obtained from The Cancer Genome Atlas (TCGA). Considering 27 groups with different cancers, about 59.26% (16 groups) of them exhibited a higher mRNA expression of *LAMC2* in tumor samples, comparatively to normal tissues (Figure 4.1 A). Regarding these 16 groups, 87.5% (14) of them showed a highly significant expression of LAMC2 (p<0.0001, for bladder urothelial carcinoma (BLCA), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), colon/rectal adenocarcinoma (COADREAD), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), lung squamous cell carcinoma (LUSC), stomach adenocarcinoma (STAD), stomach and esophageal carcinoma (STES), thyroid carcinoma (THCA) and uterine corpus endometrial carcinoma (UCEC), p=0.0378 for kidney renal papillary cell carcinoma (KIRP) and p=0.0050 for rectum adenocarcinoma (READ)). For the purpose of knowing further about the relevance of LAMC2 in uterine cervix cancer, a dataset of squamous cell carcinoma and adenocarcinoma (CESC) were analyzed. In order to get more information, data from Gene Expression Omnibus (GEO) were joined to TCGA data. The expression of LAMC2 in 332 cancer samples was significantly higher (p< 0.0001), comparatively to 27 normal uterine cervix tissues present in both databases (Figure 4.1 B). Moreover, comparing the 3 uterine cervix tumor samples that have matched non-tumor samples, we observed that LAMC2 expression also increased in tumors (p=0.0183) (Figure 4.1 C).

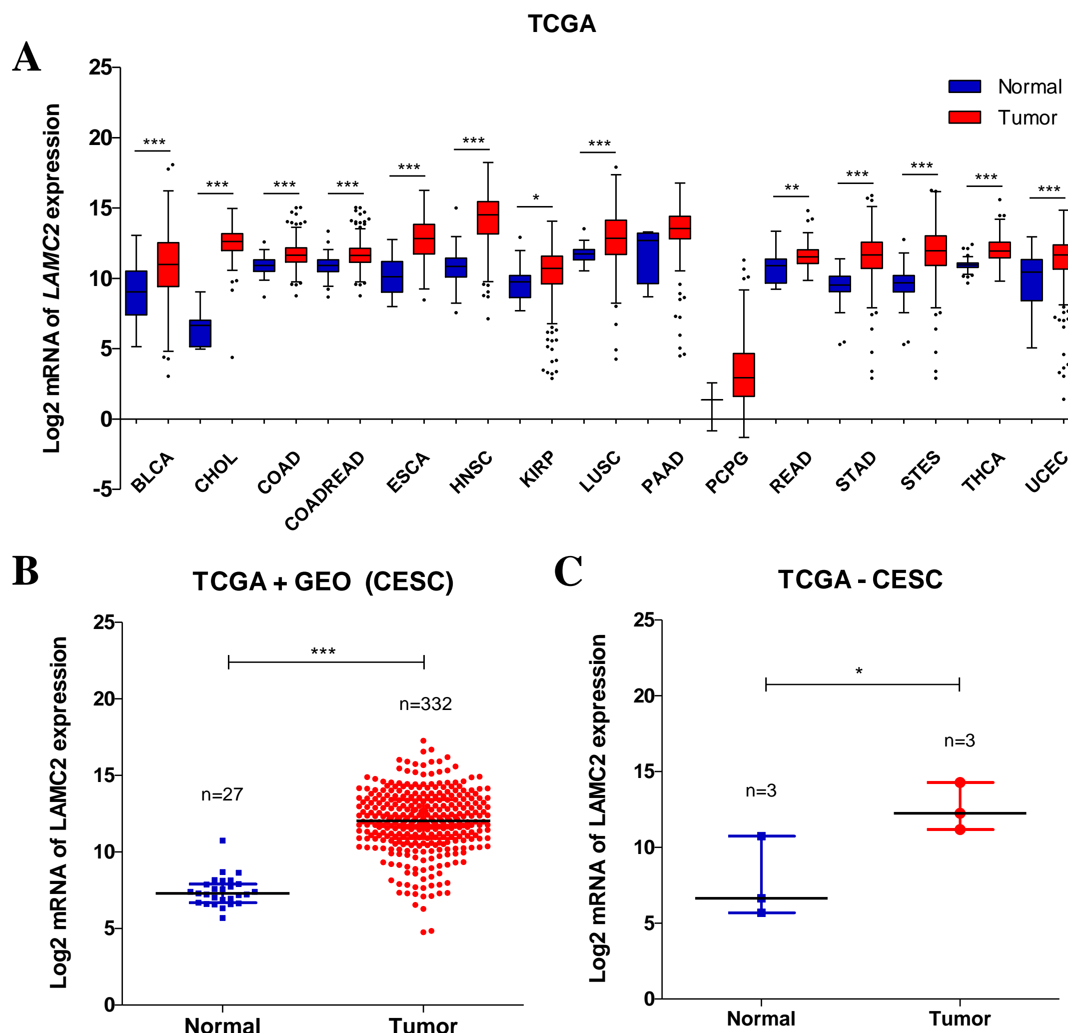


Figure 4.1 – The LAMC2 expression is significantly increased in tumor samples. Expression levels of LAMC2 by RNA--Seq data (RSEM), available in TCGA and GEO databases. **(A)** All comparable groups that showed upregulation of LAMC2 expression in tumor samples are represented on graph. Results of bladder urothelial carcinoma (BLCA), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), colon/rectal adenocarcinoma (COADREAD), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney renal papillary cell carcinoma (KIRP), lung squamous cell carcinoma (LUSC), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), rectum adenocarcinoma (READ), stomach adenocarcinoma (STAD), stomach and esophageal carcinoma (STES), thyroid carcinoma (THCA) and uterine corpus endometrial carcinoma (UCEC). **(B)** The mRNA expression of LAMC2 is upregulated in squamous cell carcinoma and adenocarcinoma (CESC) of uterine cervix tumor samples compared with the normal tissues revealed by TCGA+GEO dataset. **(C)** LAMC2 expression also is upregulated in three normal-matched tumor tissues from TCGA dataset. Results are shown as median with interquartile range. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4.2 EGF stimulates proliferation of SiHa and HeLa cells

Before addressing the role of LAMC2 in cancer features, we tried to understand if EGF was a suitable growth factor to stimulate our cell models. Hence, we addressed the EGF effect on the cell cycle by flow cytometry. Similar percentage of cells in G0/G1 were found in both cell lines in control conditions at the beginning of experience (0 h) and at 16h (Figure 4.2). However, in both cell lines the percentage of cells in G0/G1 significantly decreases ($p<0.0001$ for both cell lines) with the EGF supplementation with a consequently increase of cells in S+G2/M phases, showing that EGF stimulates cell proliferation.

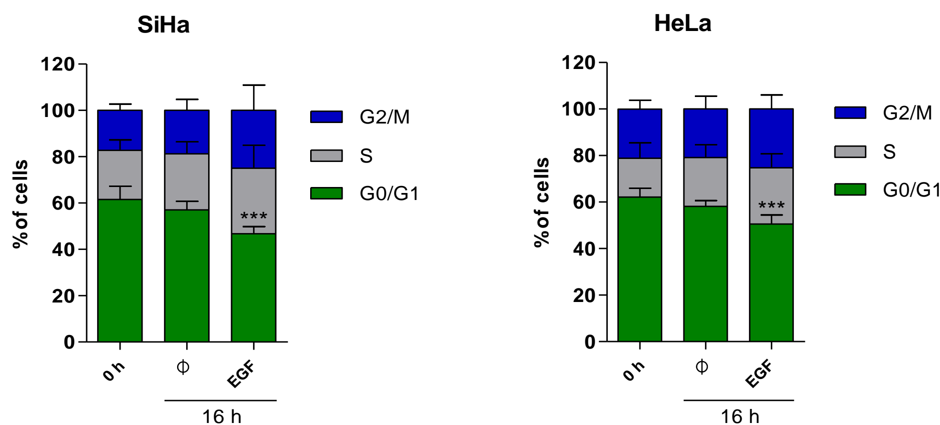


Figure 4.2 – EGF stimulates proliferation of SiHa and HeLa cells. Cell cycle analysis was performed by flow cytometry, cells were collected in three groups: controls (0h and 16 h) and 16h in EGF supplied medium. Based on DNA content histograms, the stacked bar graph represents the percentage of cells in different cell cycle phases. Both cells significantly ($p<0.0001$) responded to EGF treatment, increasing cell proliferation. Results are shown as mean ± SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4.3 EGF decreases doubling time (DT) and cell cycle duration in SiHa and HeLa cells

A cell proliferation assay was performed in order to understand how EGF treatment affects the growth population dynamics over 48 hours. For that, except to control groups, cells were supplied with EGF after 8 h on starvation and collected at 6, 12, 24, 30 and 48 hours.

Growth curves showed that cells treated with EGF proliferate slightly more compared to control groups (Figure 4.3 A and B). The population doubling time (DT) is also lower in EGF conditions, demonstrating that EGF tends to accelerate the cell cycle in both cell lines, though not in a statistical significant way (Figure 4.3 C). Furthermore, SiHa cells needed significantly ($p=0.0460$ for control and $p=0.0041$ for EGF treatment) less time to duplicate, observed by the lower DT in comparison to HeLa cells. However, there were no significant differences between treatments. Thus, only a biologic tendency of EGF to accelerate the population growth due by the increasing of cell division speed was found.

To determine how EGF could accelerate cell cycle, the duration of each cell cycle phase was calculated (Figure 4.3 D). The results showed that in both cell lines the EGF suppling interfered with the first phase of the cycle, G0/G1. Thus, the treatment significantly ($p<0.0001$ for both cell lines) decreased the time necessary to complete this phase. Due to this, the expression of possible interveners of this cell cycle phase were also analyzed. Accordingly a significant ($p=0.0001$ for SiHa and $p=0.0002$ for HeLa) increase in cyclin D1 mRNA levels was observed upon EGF exposure (Figure 4.3 E). These results indicated that the higher amounts of cyclin D1 in both cell lines, resulted of EGF stimulation, leading to the acceleration of the G0/G1, which, in turn, resulted in increased proliferation.

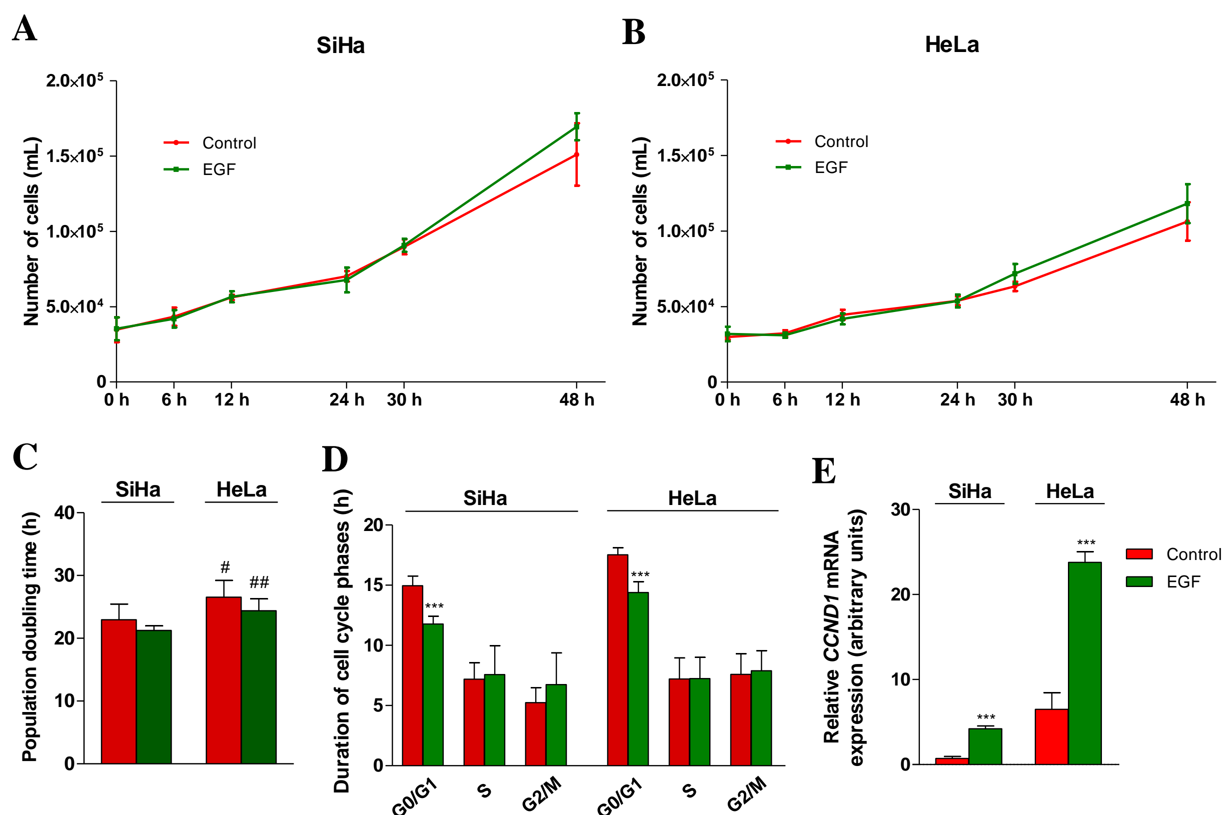


Figure 4.3 – EGF decreases doubling time (DT) and cell cycle duration in SiHa and HeLa cells. Proliferation curves, obtained by counting the number of cells, representing SiHa **(A)** and HeLa **(B)** population dynamics over 48 hours. Cells treated with EGF (green) proliferate slightly more compared to control groups (red). **(C)** Doubling time (DT) of SiHa and HeLa cells cultured in control conditions or in EGF supplied medium. EGF treatment tends to decrease DT in both cell lines. **(D)** Representation of duration of each cell cycle phases with or without EGF supplementation. G0/G1 phases were significantly accelerated ($p<0.0001$ for both cell lines). **(E)** The relative CCND1 mRNA quantification in control conditions and after EGF stimulation showed that both cell lines express higher amounts of cyclin D1 in presence of EGF. Results are shown as mean ± SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Significant differences: asterisks (*), effect of EGF treatment and hashes (#), comparison between cell lines.

4.4 Expression of *LAMC2* is upregulated by EGF

It is well known that the laminins are important proteins involved in tumor progression. However, its regulation by growth factors remains unclear, despite some studies have claimed that EGFR signaling would be responsible for LAMC2 expression [22]. We have previously demonstrated that both types of uterine cervix cancer cells respond to stimulation with EGF by increasing proliferation. To investigate the effect of growth factors on cells, cell lines were treated with EGF (25ng/mL) and were collected 16h after supplementation. The mRNA levels of the three chains of laminin-332 and the other γ chains 1 and 3 were analyzed by qRT-PCR. As shown in figure 4.4 A, the results revealed a significant (p<0.0001) differential expression of laminins in both cell lines. SiHa cells express higher levels of all analyzed laminin chains comparatively to HeLa cells. In control conditions, the α (LAMA3) and β (LAMB3) chains of laminin-332, as well as γ1 (LAMC1) and γ3 (LAMC3) chain, showed the same expression levels within each cell line (Figure 4.4 B and C). In SiHa cells, EGF treatment does not change significantly the expression of LAMA3, LAMC1 and LAMC3, however, the levels LAMB3 were significantly lower (p<0.0001) compared to the control condition (Figure 4.4 B). Regarding Hela cells, EGF stimulation does not affect the mRNA levels of LAMA3, LAMB3, LAMC1 and LAMC3 (Figure 4.4 C). Concerning LAMC2 expression, the mRNA levels were significantly (p<0.0001) up-regulated with EGF treatment, in SiHa and HeLa, representing an increase in 2.3- and 3.34-fold, respectively (Figure 4.4 B and C).

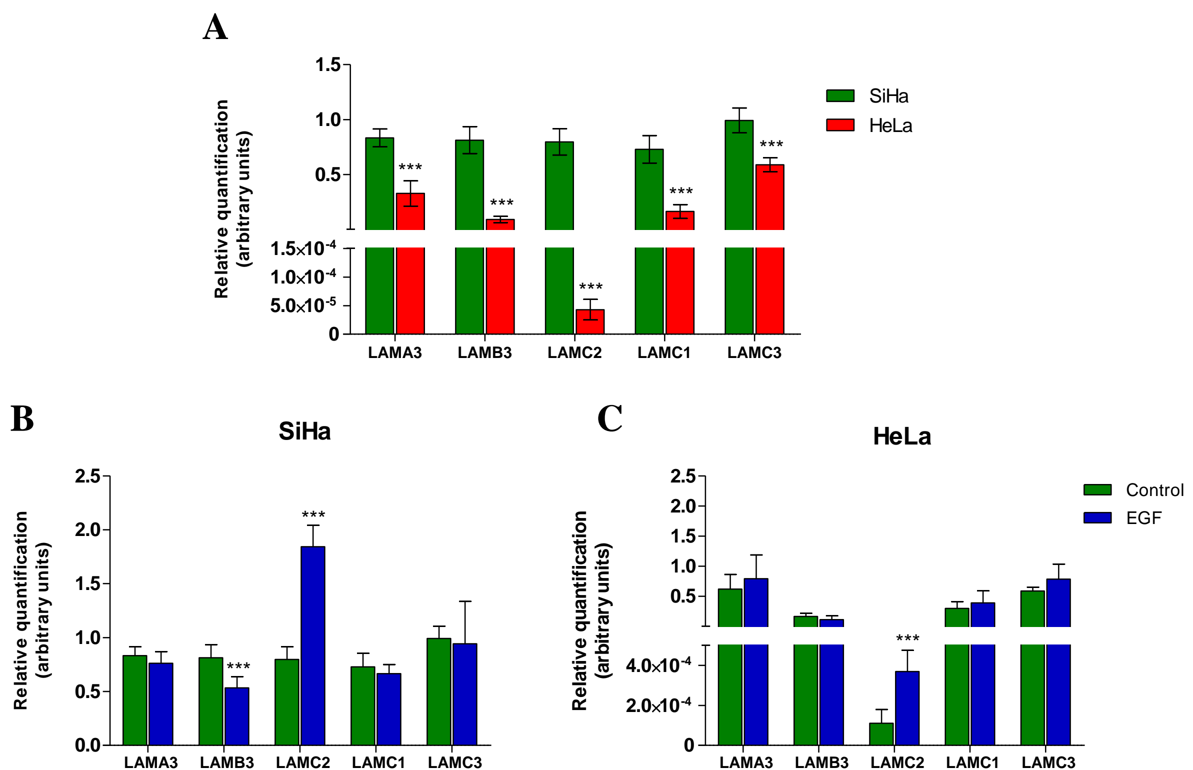


Figure 4.3 – The effect of EGF in α3, β3 and γ2 from laminin-332 and γ1 and γ3 chains expression in SiHa and HeLa cells. After 16h of EGF supplementation, the mRNA levels of different laminins were analyzed. **(A)** Comparatively, the laminins mRNA levels were higher in SiHa than in HeLa cells. **(B)** In SiHa cells, EGF treatment does not change significantly the expression of LAMA3, LAMC1 and LAMC3, but the levels of LAMC2 were significantly higher compared to the control condition. LAMB3 expression decrease in the presence of EGF. **(C)** In Hela, EGF stimulation does not affect the mRNA levels of LAMA3, LAMB3, LAMC1 and LAMC3, but LAMC2 gene were up-regulated with EGF conditions. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

4.5 EGF stimulates proliferation independently of LAMC2 in SiHa cells and more efficiently in the absence of LAMC2 in HeLa cells

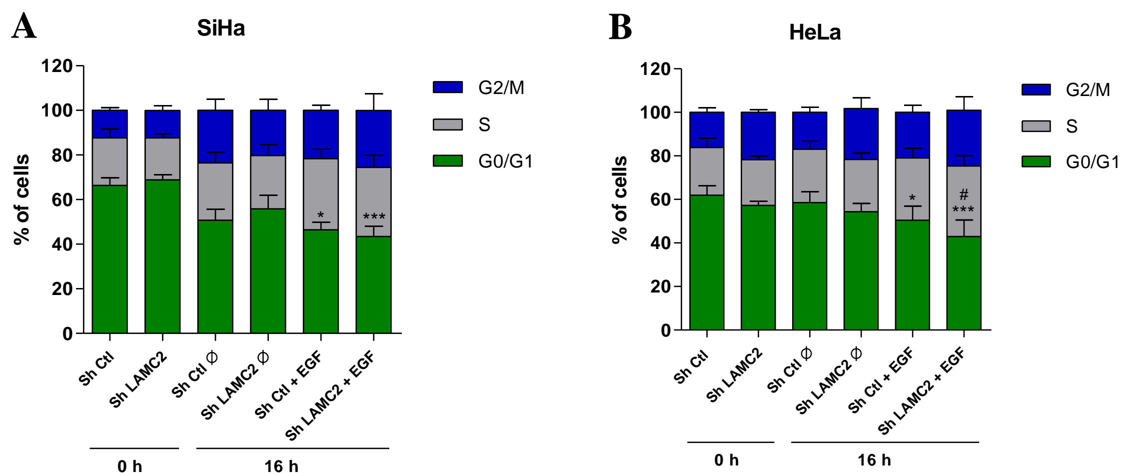
To assess the effect of LAMC2 knockdown on cells proliferation as well as to understand its influence on EGF response, this experiment was performed with shControl and shLAMC2 groups. LAMC2 knockdown was confirmed by immunofluorescence (Figure 4.9 A) The analysis of cell cycle, proliferation, calculation of population DT and duration of each cell cycle phases, were also performed.

Figure 4.5 A and B show that the percentage of cells in G0/G1 was similar in both cell lines at the beginning of the experience (0 h). However, after 16h, even in control conditions, the percentage of cells in this phase decreases in SiHa cells, and the knockdown group (ShLAMC2) seems to have a lower number of cells in S/G2-M (Figure 4.5 A). This could suggest a knockdown effect, though after EGF supplementation this difference is abolished. Regarding EGF effect, both groups, shControl (p=0.153 for SiHa) and shLAMC2 (p=0.0002 for SiHa), respond similarly to EGF treatment. Thus, EGF promotes significantly mitotic progression, but independently of LAMC2 subunit in SiHa cell line. In Hela cells, there were no differences between the beginning and the control condition, after 16 hours (Figure 4.5 B). However, in the presence of EGF both groups respond to proliferation stimulus (p=0.0122 for shControl, p=0.0010 for shLAMC2), which led to an increase of cells number in S+G2/M phases. Then again, EGF also promotes proliferation in these groups, but in HeLa cells this response was increased in the absence of LAMC2 (p= 0.0408).

The results of cell proliferation assay reveal, at the end point, that transfected cells had growth curves similar to WT cells (Figure 4.5 C and D). After 48 hours, both cell lines had the same response to EGF, which always placed the EGF proliferation curves above control ones. Thus, the EGF exposed cells proliferate slightly more in comparison to control groups. Regarding the knockdown effect in SiHa cells (Figure 4.5 C), the shLAMC2 group exposed to EGF reached the highest number of cells, showing a proliferative effect in lack of LAMC2. However, in HeLa cells the knockdown had the opposite effect in the group treated with EGF (Figure 4.5 D).

Once again, the population DT was lower in SiHa cells comparing to HeLa (Figure 4.5 E). In both cell lines, there were no significant differences between shControl and shLAMC2. However, when comparing knockdown groups, HeLa cells needed more time to double its population than SiHa cells (p=0.0129 for shLAMC2 under control conditions, p=0.0030 for shLAMC2 under EGF treatment). Nevertheless, EGF tends to accelerate population DT in both cell lines.

Regarding the duration of each cell cycle phase, results show once again, that in both cell lines the EGF supplementation significantly (p=0.0003 for SiHa shControl, p<0.0001 for the remaining groups) accelerated the first phase of the cycle, G0/G1 (Figure 4.5 F).



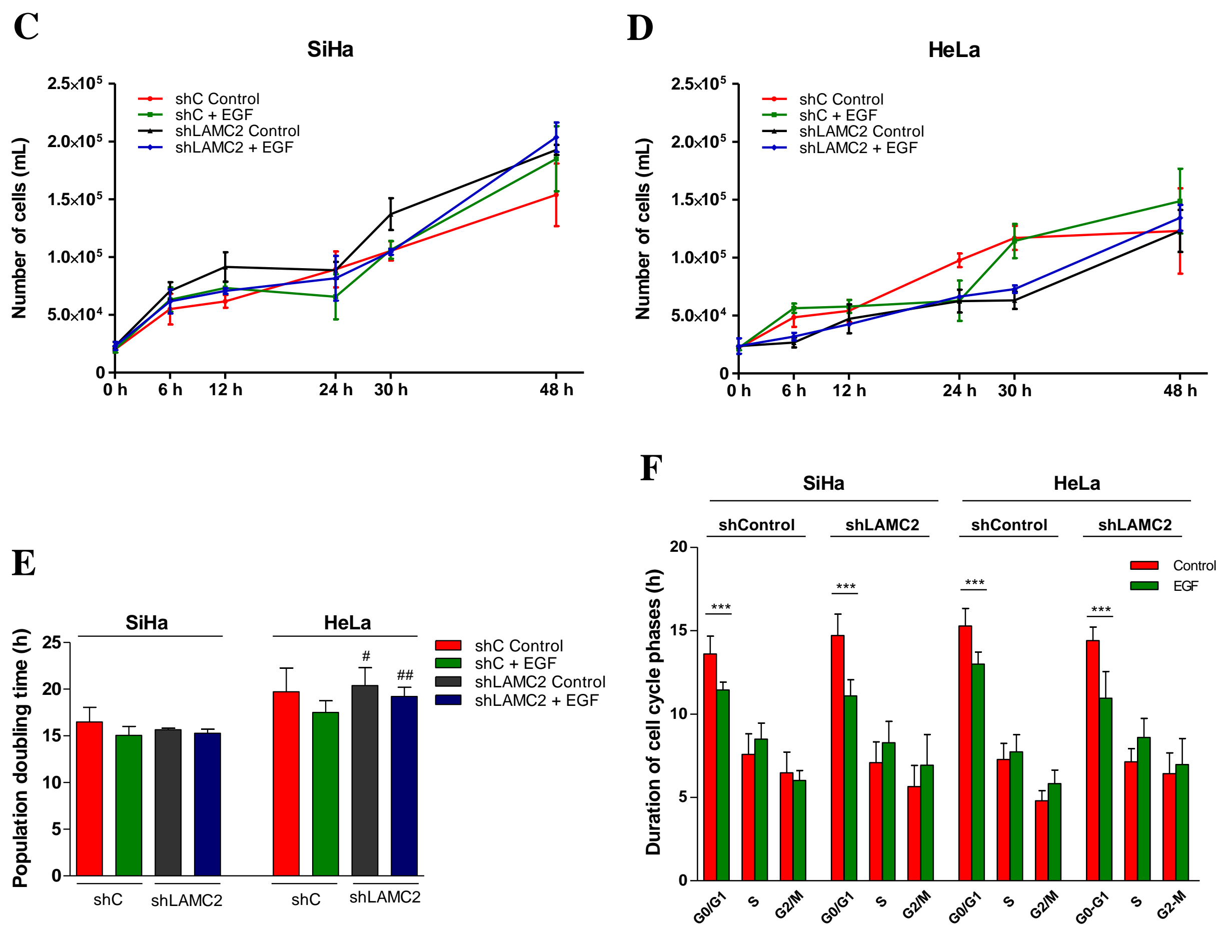


Figure 4.5 – EGF stimulates proliferation independently of LAMC2 in SiHa cells and more efficiently in the absence of LAMC2 in HeLa cells. Cells were collected in three groups: at 0h and at 16 h in EGF supplied medium or in regular medium. For each condition two groups were analyzed: shControl and shLAMC2, in order to figure out the role of LAMC2. Bar graph represents the percentage of cells in different cell cycle phases. Both cell lines, SiHa **(A)** and HeLa **(B)** responded to EGF treatment, increasing cell proliferation. However, in Hela cells the lack of LAMC2 increased this effect. **(C and D)** Growth curves representing SiHa and HeLa population dynamics over 48 hours respectively. Both cell lines had the same response to EGF, which leads to a slightly higher growth compared to controls. **(E)** Doubling time (DT) of shControl and shLAMC2, per each cell line, cultured in control conditions or in EGF supplied medium. EGF treatment tends to decrease DT in both cell lines, even within the groups. The knockdown of LAMC2 exacerbates the differences between cell lines. **(F)** Representation of duration of each cell cycles phases, which shows an acceleration of G0/G1 cell cycle phase. Results are shown as mean ± SD. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Significant differences: asterisks (*), effect of EGF treatment and hashes (#), comparison between cell lines.

4.6 Silencing of LAMC2 suppresses SiHa migration and matrix invasion

In order to investigate the *in vitro* effects of *LAMC2* in uterine cervix cancer cell migration and invasion a wound healing and transwell migration assays were performed. The experiment evolution was monitored at the following time points: 0, 6, 24, 32 and 48 hours. SiHa cells expressing LAMC2 (WT and ShControl) were able to close the wound only in the presence of EGF (Figure 4.6). However, shLAMC2 SiHa cells were not able to migrate and close the wound even in the presence of EGF. In HeLa cells, the migration capacity of cells was not dependent on neither EGF treatment nor LAMC2 expression (*supplementary Figure 1*). Therefore, silencing of LAMC2 significantly inhibited SiHa but not HeLa cells migration. Additionally, EGF stimulus is necessary to promote the matrix invasion by SiHa cells (Figure 4.7). However, the lack of LAMC2 chain inhibits the EGF effect by suppressing SiHa

cells invasive capacity. All together these results demonstrate that LAMC2 is crucial for SiHa cells migration and invasion.

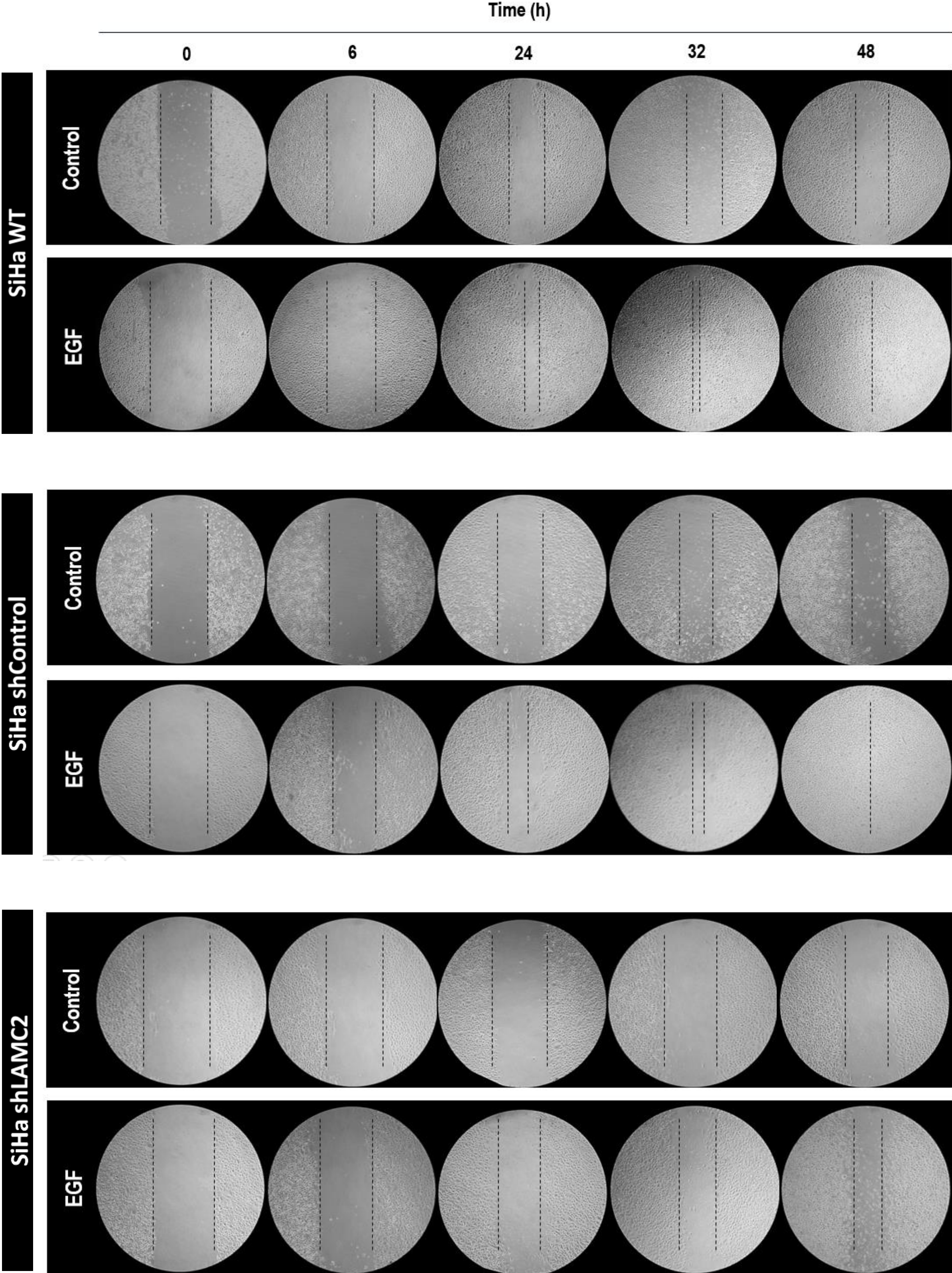


Figure 4.6 – LAMC2 is crucial for SiHa cells migration. Comparison between WT SiHa cells, shControl and shLAMC2, at 0, 6, 24, 32 and 48 hours, with and without EGF. In knockdown group of SiHa cells, depletion of LAMC2 leads to an incapacity of cells to migrate even in the presence of EGF. Phase microscopy (original magnification: 200x).

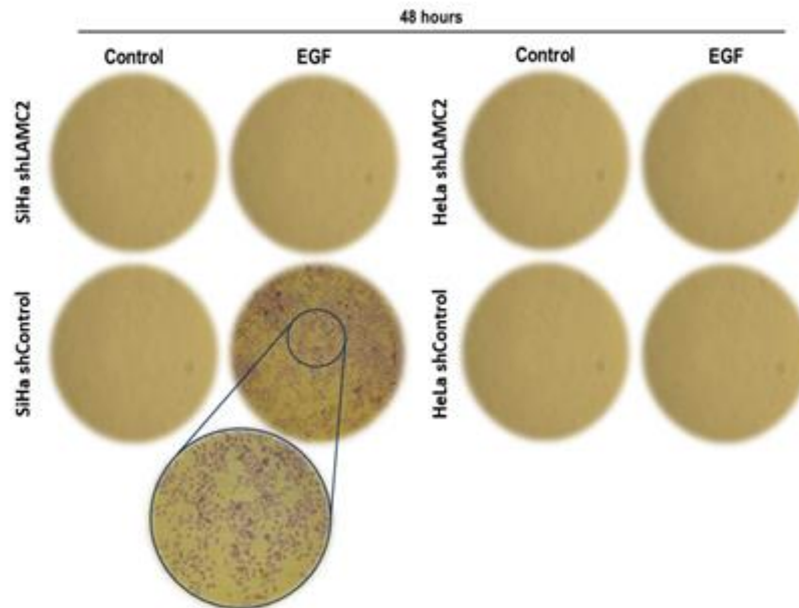


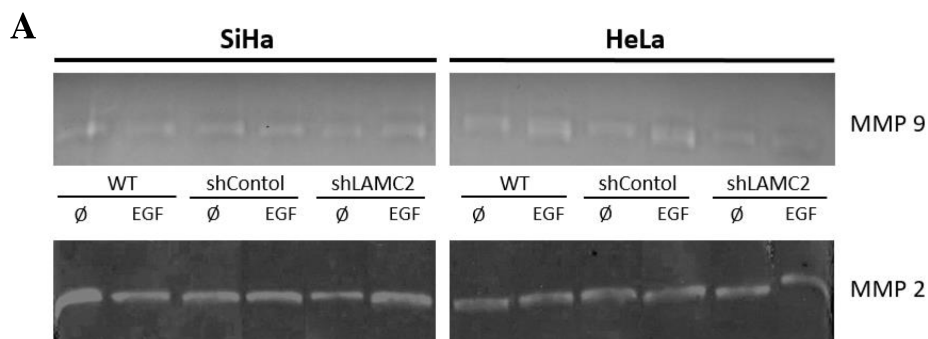
Figure 4.7 – LAMC2 is crucial for SiHa cells migration and invasive capacity. Cells were stained with cristal violet. EGF stimulus is necessary to induce invasion of SiHa cells (left down). However, the lack of chain abolishes the EGF effect. Thus, the silencing of LAMC2 suppressed SiHa cell invasion *in vitro.* In HeLa cells, both EGF treatment and knockdown of LAMC2 had no effect on invasion.

4.7 MMP activity is increased by EGF

In order to analyze the MMPs activity a zymography assay was performed. After 16h of supplementation with EGF, the media supernatant of each cell line was collected and concentrated (Figure 4.8 A).

Regarding the MMP9 activity in SiHa cells, the results suggest that under EGF conditions, the shControl and shLAMC2 samples, decreased and increased, respectively, (Figure 4.8 B). In HeLa cells, the results also show an increase of MMP9 activity in the samples under EGF treatment. Besides that, the LAMC2 knockdown decreased MMP9 activity in both cell lines, compared to WT and shControl groups. Moreover, this enzyme activity did not change upon EGF stimuli, in both WT cells.

Relatively to MMP2 in SiHa cells, the knockdown led to a slight decrease. However, the EGF stimulation restores the enzyme activity, being the only group that had responded positively to EGF supplementation (Figure 4.8 C). In Hela cells, the MMP2 activity showed an increase in shLAMC2 group in control conditions. The EGF treatment led to an increase of MMP2 in HeLa WT, whereas in shControl and shLAMC2 a decrease was observed.



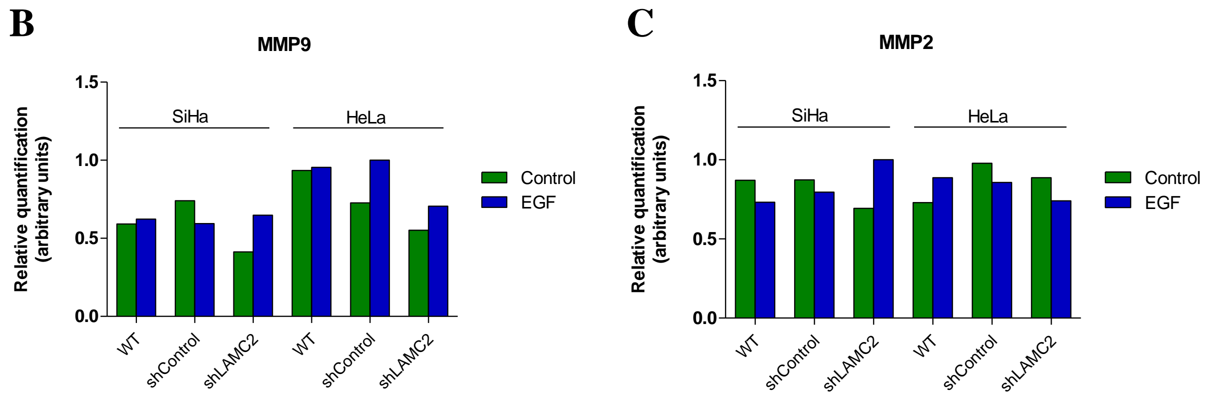
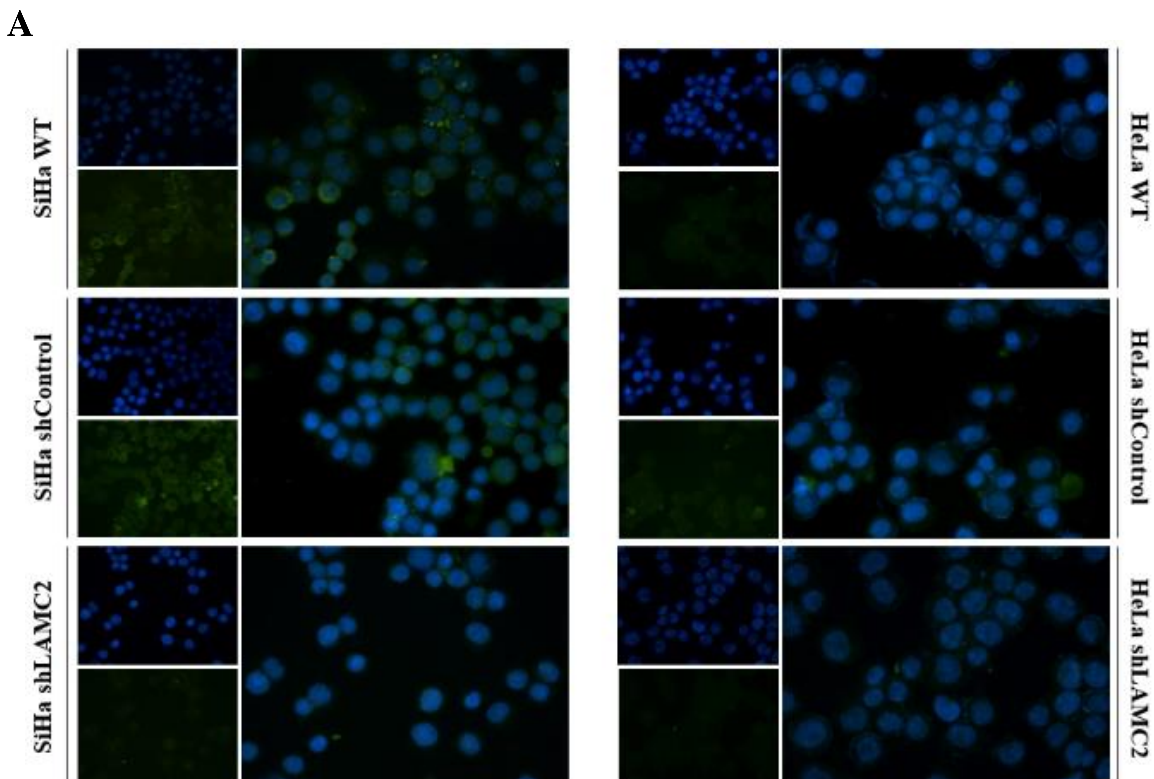


Figure 4.8 – Zymography of SiHa and HeLa media supernatants. **(A)** Zymogram for WT, shControl and shLAMC2 groups of each cell line with or without (|Ø) EGF supplementation. **(B)** Quantification of each band detected for MMP9 in all groups of both cell lines. **(C)** Quantification of each band detected for MMP2.

4.8 Knockdown LAMC2 chain affects the expression of LAMA3 and LAMB3 in SiHa and HeLa, at the protein level

To investigate the role of LAMC2 in uterine cervix carcinoma, was performed a silencing of *LAMC2* expression through shRNA transfection assay. The protein abundances were analyzed by immunofluorescence, using specific antibodies against target proteins, in three groups: WT, shControl and shLAMC2. Figure 4.9 A shows the effect of LAMC2 chain knockdown on laminin-332 chains, in both cell lines, SiHa and HeLa. Compared to control group, a significant decrease (p<0.0001 for both cell lines) of LAMC2 protein was detected in both knockdown cell lines, proving the efficacy of LAMC2 silencing (Figure 4.9 A). Regarding LAMA3, in SiHa and HeLa, LAMC2 knockdown led to a significant decrease (p<0.0001 for both cell lines) compared to control cells (Figure 4.9 B). Figure 4.9 C shows the immunofluorescence of LAMB3, where the knockdown of LAMC2 did not affect its expression in SiHa and HeLa cells, as compared to shControl. However, in transfected cells of both cell lines, the LAMB3 expression decreases. These results also show that SiHa cell line express higher basal levels of LAMB3 and LAMC2, when compared to HeLa cells.



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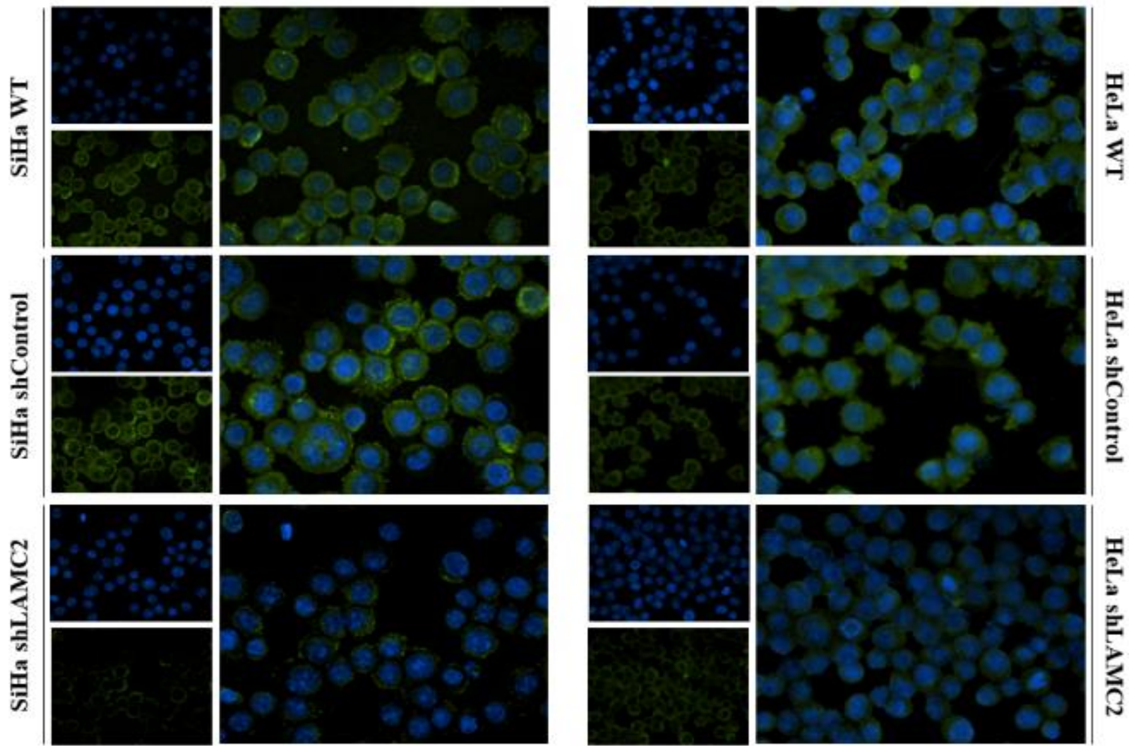

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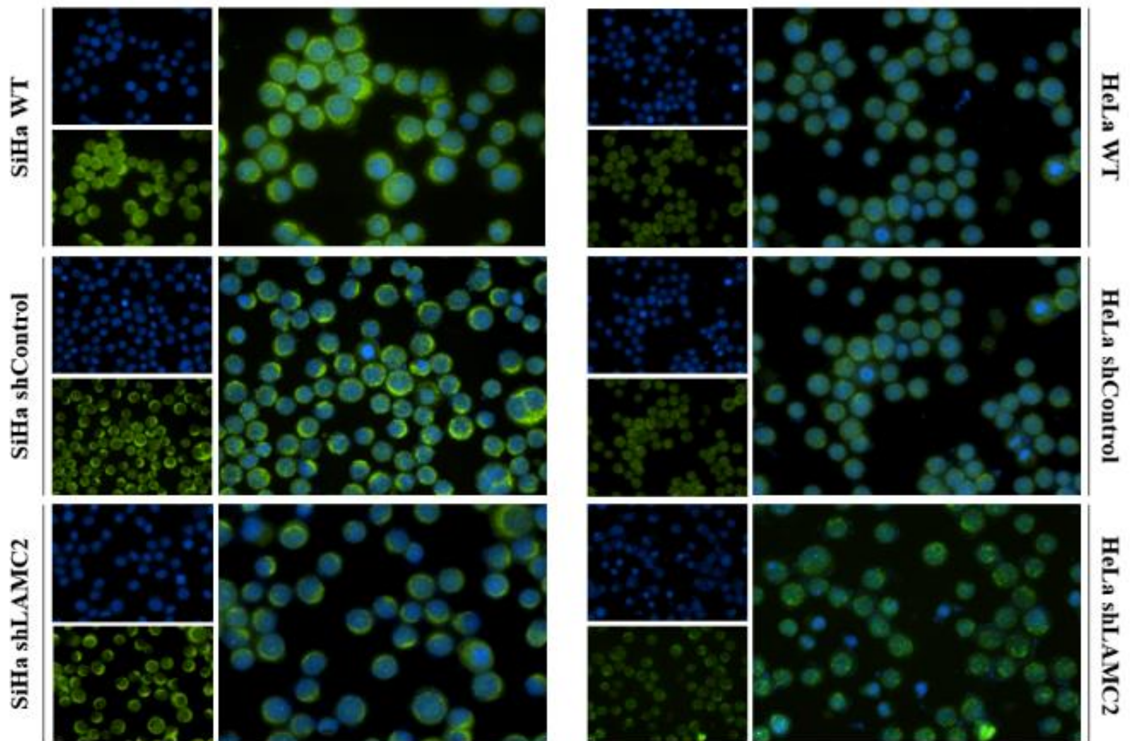


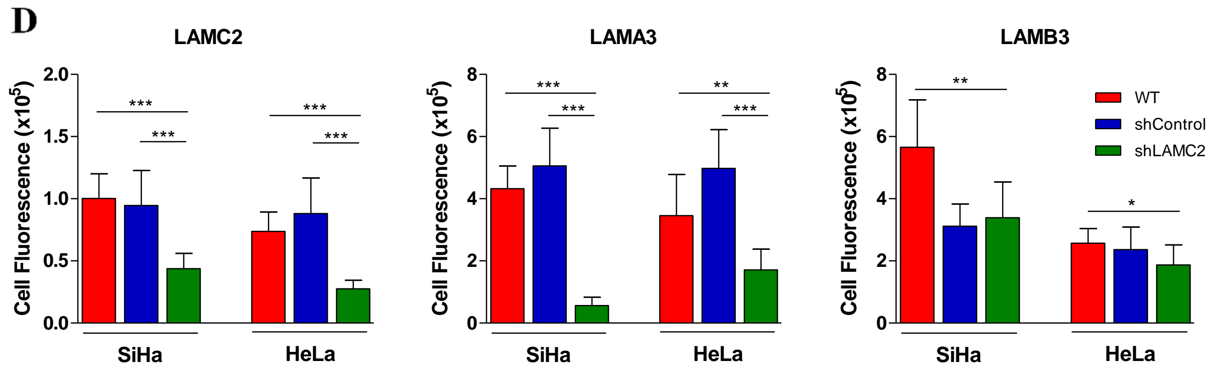


Figure 4.9 – Knockdown of LAMC2 chain affects the expression of LAMA3 and LAMB3 in SiHa and HeLa. Comparison between controls and transfected cells. **(A)** The result shows an efficient LAMC2 down-regulation after its knockdown with shRNA. **(B)** LAMA3 expression decreased in knockdown cells. **(C)** After knockdown of LAMC2, the LAMB3 expression also decreased in HeLa. **(D)** Graphs of LAMC2 (left), LAMA3 (middle) and LAMB3 (right) immunofluorescence quantification (CTCF) using ImageJ program. Fluorescence microscopy (original magnification: 200 x). Nuclei were stained with DAPI (blue). Results are shown as mean ± SD. **p*<0.05, ***p*<0.01, ****p*<0.001.

4.9 Knockdown LAMC2 chain increases the expression of LAMC1 and in SiHa, at the mRNA level

To find out the effect of LAMC2 knockdown on the expression of all laminin γ chains, a qRT-PCR assay was performed. The relative mRNA expression levels of each group, shLAMC2 and ShControl, was determined in the presence or absence of EGF.

In order to confirm the knockdown effect of shLAMC2, the LAMC2 mRNA levels were also quantified. Surprisingly, shLAMC2 SiHa cells express higher levels of LAMC2 mRNA than shControl cells (*p*=0.0003) (Figure 4.10 A). It was also observed that EGF treatment strongly stimulated the LAMC2 transcription in SiHa cells in shControl (*p*=0.0003) and in shLAMC2 (*p*<0.0001) SiHa cells. In HeLa cells, the LAMC2 mRNA levels were lower in shLAMC2 cells than in shControl cells in all culture conditions (*p*=0.0326 for control) (Figure 4.10 B). The EGF treatment also stimulated the LAMC2 expression in shControl compared to control conditions (*p*=0.0248).

In order to study the effect of knockdown on regulation of the expression of others γ chains, the mRNA levels of LAMC1 and LAMC3 genes were also analyzed. The LAMC1 gene expression in SiHa cells was significantly increased in shLAMC2 cells in both conditions (*p*=0.0009 for control, *p*=0.0017 with EGF), comparing with shControl cells (Figure 4.10 C). In HeLa cells, shLAMC2 cells express lower levels of LAMC1 (*p*=0.0056 for control, *p*=0.0002 with EGF) (Figure 4.10 D). The results of LAMC3 expression in SiHa showed an increase due to LAMC2 knockdown (*p*=0.0177), however the LAMC3 mRNA levels were not changed due to the effect of EGF (Figure 4.10 E). In Hela cells, no alterations were observed in LAMC3 mRNA levels related to LAMC2 knockdown, but EGF induced the decrease of LAMC3 mRNA levels in shLAMC2 cells (*p*=0.0057 with EGF) (Figure 4.10 F). From these results it was interesting to see that in SiHa cells the knockdown of LAMC2 induced the increase of the expression of the other γ chains, LAMC1 and LAMC3.

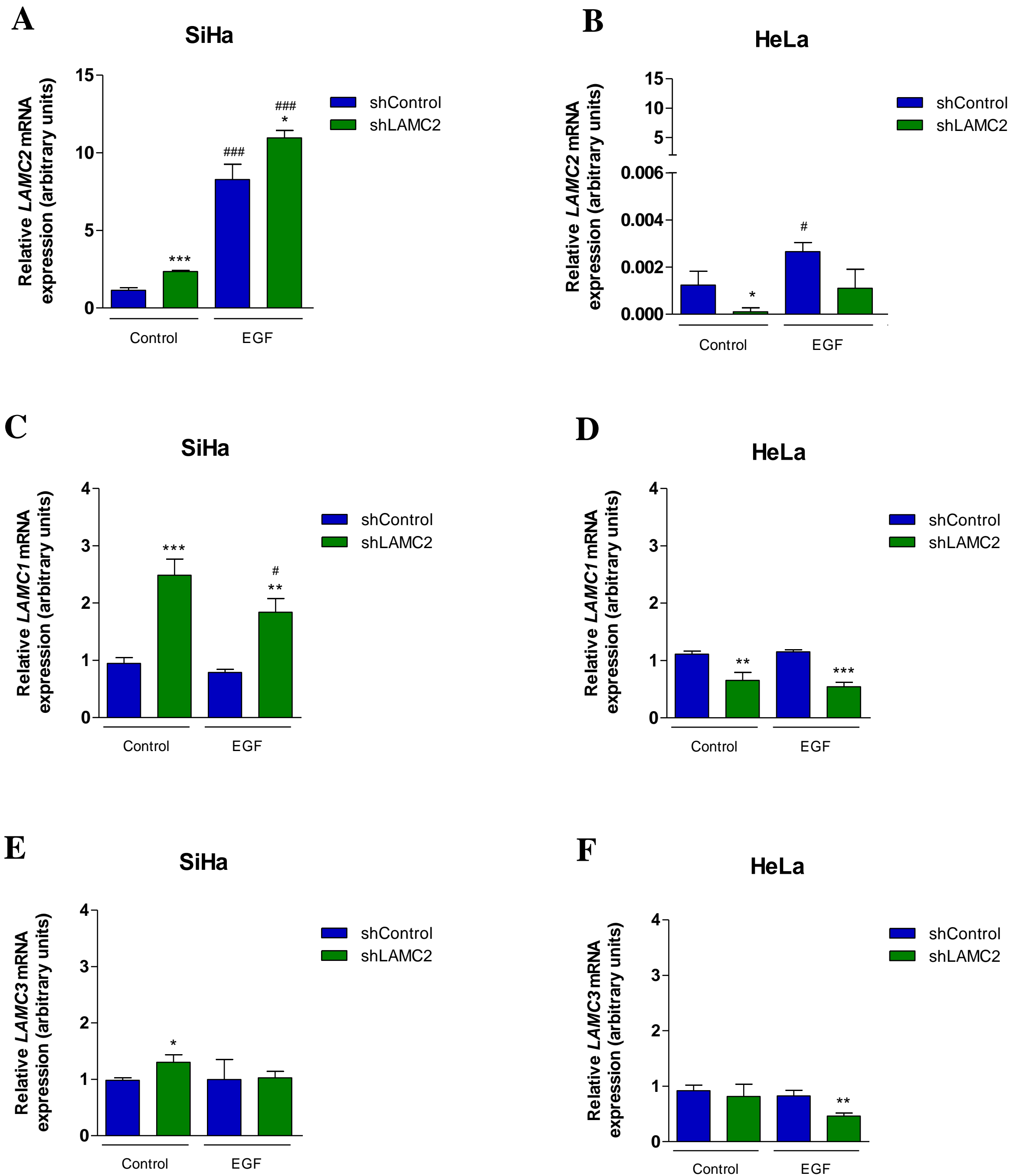


Figure 4.10 – In SiHa cells the knockdown of LAMC2 induced the increase of the expression LAMA3, LAMB3, LAMC1 and LAMC3. Results of mRNA levels by qRT-PCR assay of LAMA3, LAMB3, LAMC2, LAMC1 and LAMC3 expression after LAMC2 knockdown. The mRNA expression was measured in the presence or absence of EGF. **(A)** In SiHa cells, shLAMC2 cells express higher mRNA levels of LAMC2 and EGF treatment stimulated the LAMC2 expression. **(B)** In HeLa cells, the LAMC2 expression decreased by knockdown in all culture conditions Once again, the EGF treatment stimulated the LAMC2 expression. **(C)** In SiHa, the LAMC1 gene expression was increased in shLAMC2 cells in all groups. **(D)** In HeLa cells, shLAMC2 cells express lower levels of LAMC1. **(E)** The results of LAMC3 expression in SiHa showed an increase due by LAMC2 knockdown. **(F)** In Hela cells, no alterations were observed in LAMC3 mRNA levels related to LAMC2 knockdown. Results are shown as mean ± SD. **p*<0.05, ***p*<0.01, ****p*<0.001. Significant differences: asterisks (*), effect of LAMC2 knockdown and hashes (#), effect of growth factors stimulation.

4.10 Pulse chase analysis of *LAMC2* expression proves the efficacy of shLAMC2

The pulse chase assay was performed to verify the role of LAMC2 silencing on its mRNA expression levels over time, after the unexpected increase of LAMC2 mRNA levels, in the previous result. Cells were collected at 0, 16, 18, 20, 22, 24 and 26 hours. Once again, it was clear the higher expression level of LAMC2 in SiHa (Figure 4.11 A) when compared to HeLa (Figure 4.8 B). In the control group, the EGF supplementation results in a higher gene expression of LAMC2, especially at 18 hours for SiHa and 16 hours for HeLa. Regarding knockdowns, the expression was lower in both cells, in comparison to shControl group. Therefore, this results show that the silencing of LAMC2 led to a decrease in their mRNA levels.

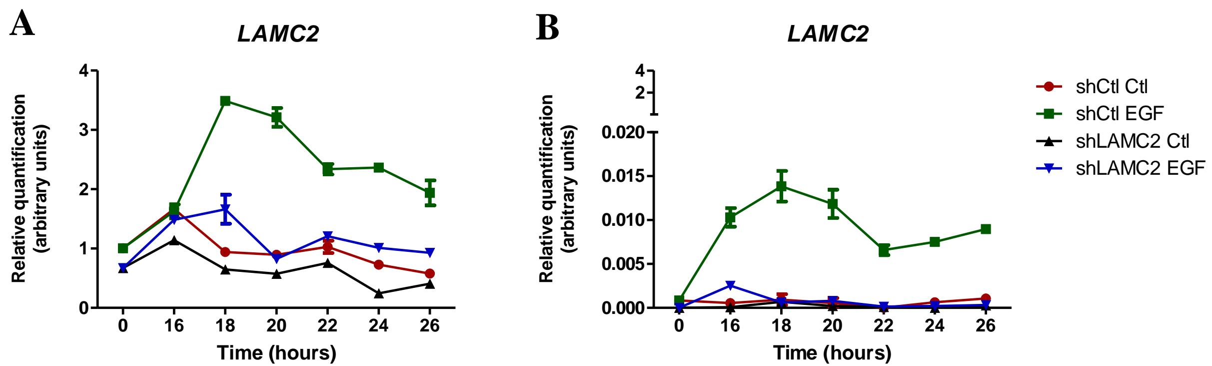
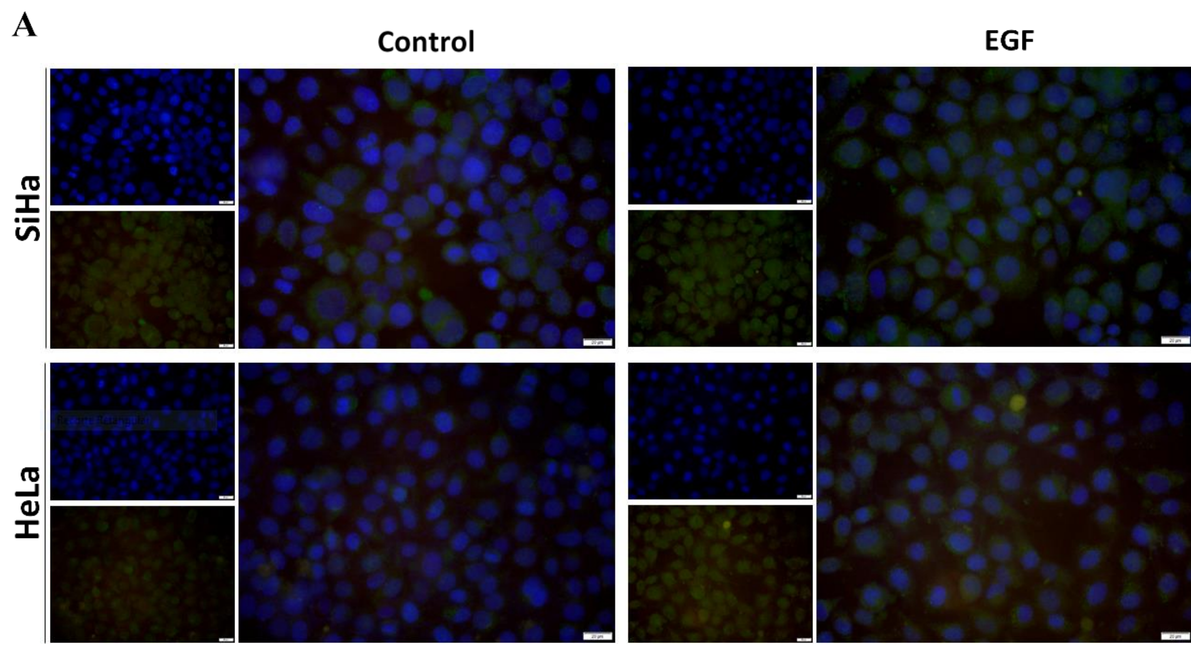


Figure 4.11 – Pulse chase assay in SiHa and HeLa cells of LAMC2 expression proving knockdown efficacy. In control groups, the mRNA levels of LAMC2 expression increased after EGF stimulus in both cell lines, SiHa (**A**) and HeLa (**B**). The pulse chase graphs of knockdown groups show the efficient LAMC2 silencing.

4.11 LAMC1 expression is upregulated by EGF in Hela but not in SiHa

As it was verified LAMC2 knockdown induces the transcription of *LAMC1* and *LAMC3*. However, this effect was significantly higher for *LAMC1* and it continued even in the presence of EGF stimuli. Thus, we evaluated the expression of LAMC1 at the protein level by immunofluorescence, also addressing the role of EGF, in wild type (WT) cell lines. The result of LAMC2 staining again confirmed that EGF supplementation significantly induced an upregulation of its levels in both cell lines (p=0.0453 for SiHa, p=0.0002 for HeLa) (Figure 4.12 A and C). In SiHa a 1.4-fold increase was observed in response to EGF exposure, whereas in HeLa the increase was 2.3-fold. Regarding LAMC1, SiHa express higher basal levels than HeLa, however in SiHa cells no significant alterations were observed with EGF (Figure 4.12 B and D). In contrast, in HeLa cells, the stimulation with EGF led to a significant increase of LAMC1 protein levels (p=0.0180).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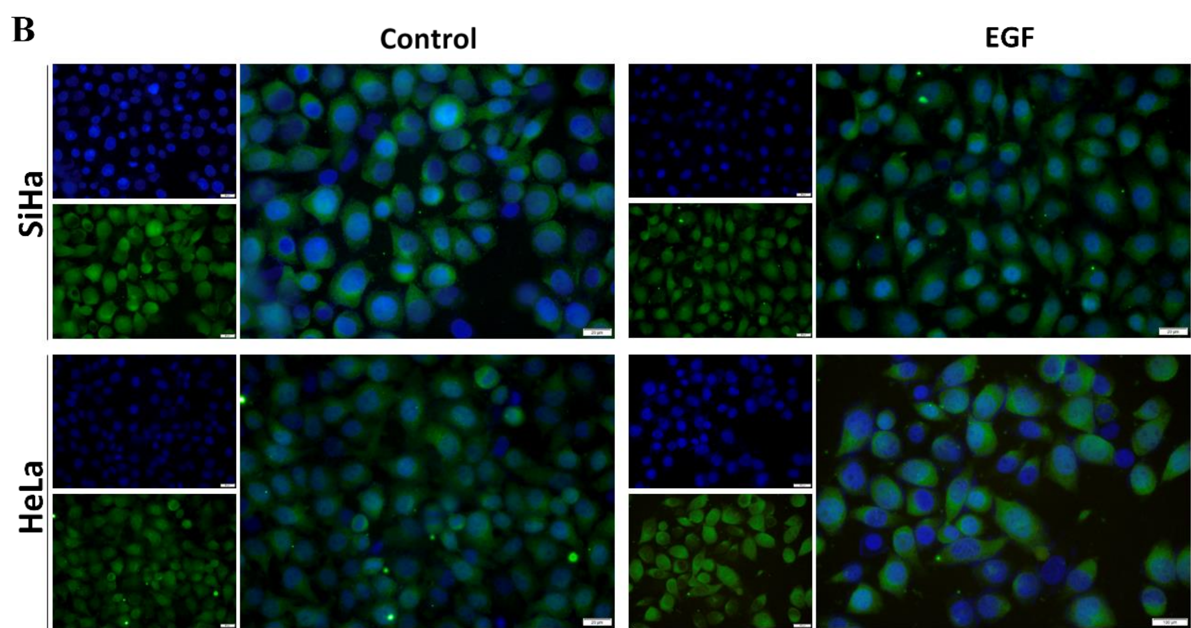
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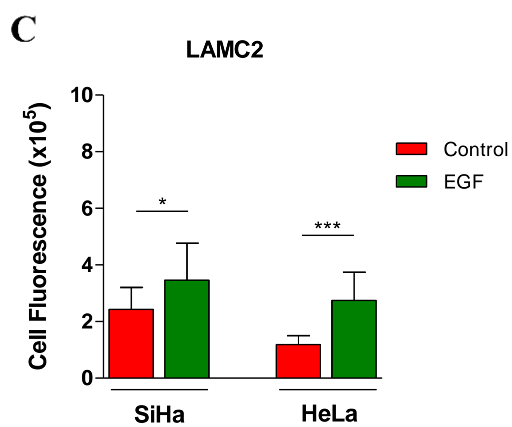
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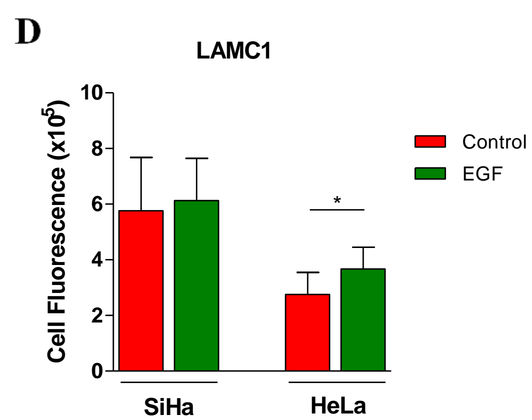
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Figure 4.12 – LAMC1 expression is upregulated by EGF in Hela but not in SiHa. Comparison between control conditions and EGF supplemented medium. **(A)** The result shows an upregulation of LAMC2 protein levels with EGF exposure in both cell lines. **(B)** In HeLa cells, the LAMC1 expression was increased by EGF stimulation, whereas did not changed in SiHa cells. Graphs of LAMC2 **(C)** and LAMC1 **(D)** immunofluorescence quantification (CTCF) using ImageJ program. Fluorescence microscopy (original magnification: 400 x). Nuclei were stained with DAPI (blue). Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

4.12 EGF modulates the FOXM1 and STAT3 binding to LAMC2 and LAMC1

As it was observed that EGF increases the expression of LAMC2 and LAMC1, we attempt to verify which were the effectors of these regulations. Recently, it has been shown that the Forkhead box M1 (FOXM1) can be a STAT3 target and its overexpression has been associated to uterine cervix carcinogenesis as well as cancer progression[6]. To disclose the role of FOXM1 and STAT3 in LAMC2 and LAMC1 regulation, upon EGF stimuli, the relative occupancy of FOXM1 and STAT3 on the LAMC2 and LAMC1 promoters were measured. These transcription factors were immunoprecipitated by ChIP assay and then a qRT-PCR was performed for the linked DNA fragments. In SiHa cells, the EGF treatment led to a significantly increase of FOXM1 binding to the promoters of both LAMC2 (p=0.0346) (Figure 4.13 A) and LAMC1 (p=0.0047) (Figure 4.13 B), whereas for STAT3 the opposite effect was observed. In Hela cells, the EGF supplementation led to an increase of FOXM1 and STAT3 binding to LAMC2 and LAMC1 promoters.

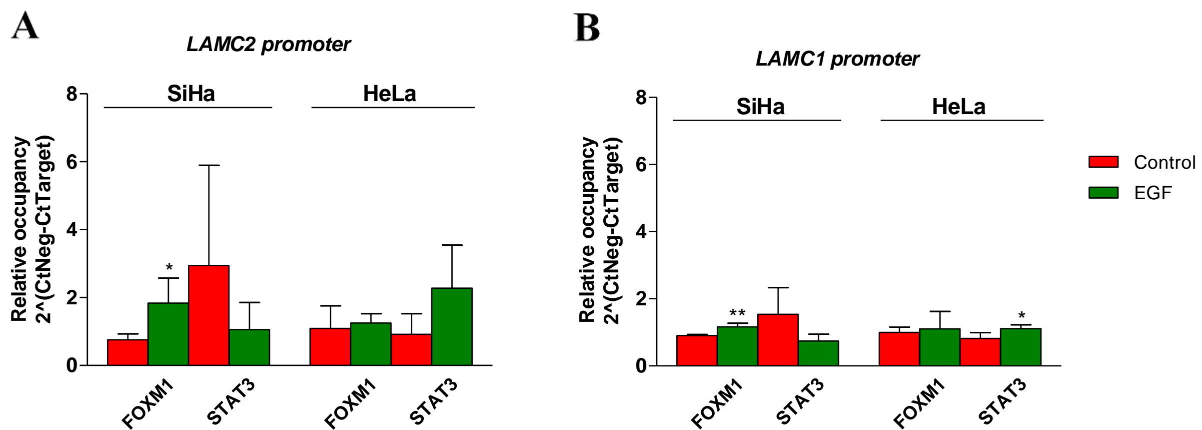

Figure 4.13 – EGF modulates the FOXM1 and STAT3 binding to *LAMC2* and *LAMC1* promoter regions. (A) Results of *LAMC2* promoter, which shows an increase of relative occupancy of FOXM1 but not STAT3 in EGF conditions in SiHa. In HeLa, EGF stimulates STAT3 binding to LAMC2 promoter. **(B)** Results for *LAMC1* promoter, showing an increase of FOXM1 binding in SiHa and an increase in STAT3 binding in HeLa, after EGF treatment. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

4.13 LAMC1 expression is controversial in uterine cervix cancer

With the intent of knowing further about the relevance of LAMC1 in uterine cervix cancer, a dataset of squamous cell carcinoma and adenocarcinoma (CESC) from TCGA data were analyzed. The expression of LAMC1 in 304 cancer samples was significantly lower (p=0.0284), comparatively to 3 normal uterine cervix tissues present at data (Figure 4.14 A). In order to get more information, especially for normal samples, data from GEO were also analyzed. GEO database showed opposite results, as the LAMC1 expression in 28 cancer samples was significantly higher (p<0.035), than 24 normal uterine cervix tissues (Figure 4.14 B). By joining the two databases, it was verified that the expression of LAMC1 continue to be significantly higher in tumor samples (p< 0.0001), comparatively to 27 normal uterine cervix tissues present in both databases (Figure 4.14 C).

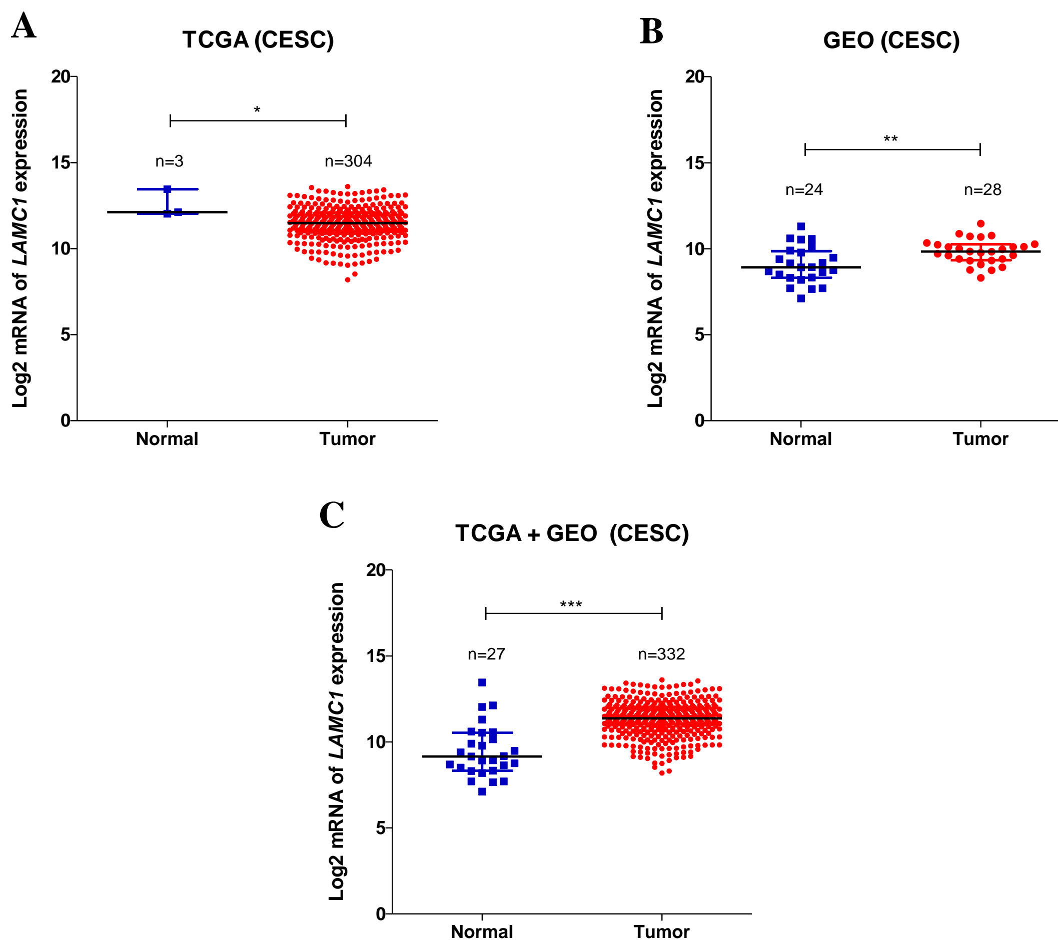

Figure 4.14 – The dynamics of LAMC1 expression is controversial in uterine cervix cancer. Expression levels of LAMC1 by RNA-Seq data (RSEM), available in TCGA and GEO databases. **(A)** The mRNA expression of LAMC1 is downregulated in uterine cervix tumor samples from TCGA, compared with the normal tissues. (B) The mRNA data from GEO database showed an increase of LAMC1 expression in cancer samples. (C) The mRNA levels of LAMC1 that result of joining the two data bases, showing that the LAMC1 expression is higher in tumor samples comparatively to 27 normal uterine cervix tissues present in both databases. Results are shown as median with interquartile range. *p<0.05, **p<0.01, ***p<0.001.

5. Discussion

In 2011, Hanahan & Weinberg, described two new emerging hallmarks based on tumor microenvironment network formation and their signaling interactions, which are essencial for the acquisition of the six issential cancer hallmarks, sustaining proliferative signaling, evasion to growth suppressors, cell death resistance, replicative immortality, angiogenesis capacity, and increase invasive and metastasis ability [73]. ECM is the major component of microenvironment, being composed by a complex network of macromolecules. The disorganization of this network and alterations in its composition lead to a generation of a tumorigenic microenvironment [12,13].

Laminin-332 is one of the most abundant components of BM present in ECM. Several studies have presented laminin-332 as a highlight subject. High expression level of this proteins were related to tumor progression [17,23]. The main objective of this thesis was to study the role of the LAMC2 chain of laminin--332 in tumor development and progression under the stimulation of EGF, which is considered the main growth factor orchestrating the uterine cervix carcinogenesis [53].

Based on the literature, our first approach was to investigate the expression of this chain of laminin--332 within different types of carcinomas, using patient's data. For this propose, TCGA database was accessed and it was found that the expression of this protein is upregulated in at least 16 types of cancer, as shown in figure 4.1 A. This represents 59.26 % of all analyzed cancer groups with matched normal samples presented in this database, being this increase statistically significant in 87.8% of cancer types. In fact, this corroborates the results of several studies, which also found an upregulation of LAMC2 in adenocarcinomas of the stomach (STAD) [26], colon (COAD) [27], pancreas (PAAD) [28], thyroid (THCA) [29], and colorectal (COADREAD) [31], squamous cell carcinomas of the esophagus (ESCA) [34], head and neck (HNSC) [37], and lung (LUSC) [37], and bladder urothelial carcinoma (BLCA) [38]. Moreover, other cancer types that are not presented in this dataset, as lung adenocarcinoma (LUAD) [32], skin cutaneous melanoma (SKCM) [36] and squamous cell carcinoma of tongue [30], are also described as having LAMC2 upregulation. Although, there are some exceptions, both laminin-332 and LAMC2 expression has been reported to be downregulated in invasive breast [74] and prostate cancers [75]. These reports are in agreement with our findings from TCGA data of breast invasive carcinoma (BRCA) and prostate adenocarcinoma (PRAD) (data not shown). Nevertheless, alterations in LAMC2 expression were not described yet in some types of cancer (cholangiocarcinoma, kidney carcinoma, adenocarcinoma of rectum and uterine corpus endometrial carcinoma), which may open new research fields. Overall, the general consensus in the field is that in most cancer types LAMC2 is upregulated and is frequently found at the invasive front of tumors.

Skyldberg et al. (1999) and Imura et al., (2012) described LAMC2 as a marker of invasiveness in uterine cervix squamous cell carcinoma and in uterine cervix adenocarcinomas [33,41]. As expected, data from TCGA and GEO databases showed a significant increase of LAMC2 expression in uterine cervix cancer samples (Figure 4.1 B and C). Unfortunately, it was not possible to evaluate in separate the uterine cervix squamous cell carcinomas from adenocarcinomas, since this identification was not available.

Growth factors assume an important role in tumor initiation and currently, it is well established that EGF stimulates cell growth, differentiation, survival and decreases apoptosis through binding to its receptor EGFR, which can serve as an oncoprotein since it is frequently overexpressed or mutated in human cancer [48]. We assessed the EGF role on cell proliferation by a cell cycle assay in SiHa and HeLa cells. The results show a significant decrease of cells at G0/G1 phase and consequently increase of cell number at S+G2/M phases, showing that EGF really activates cell cycle in uterine cervix cancer cells (Figure 4.2). This corroborates previous studies that shows an increase cell proliferation through EGFR

activation in uterine cervix cancer cell lines, as HeLa [52,76] and CaSki cells [77]. Unfortunately, we were not able to confirm yet the expression and the activation status of EGFR, but it will be addressed soon.

Regarding proliferation curves, SiHa cells are more proliferative than HeLa cells, but upon EGF stimuli both cell lines increase slightly the proliferation rate (Figure 4.3 A and B). The effect of EGF on SiHa and HeLa cells was more evident in cell cycle analysis, in which a decrease in G0/G1 population with a concomitant increase in S+G2/M population together with a decreased DT was observed. As mentioned, we did not evaluate the expression of EGF receptors in this study, but it was described that SiHa and HeLa cells exhibit similar levels of EGFR expression [78]. It was even described that SiHa cells were slightly growth stimulated by EGF, resulting in EGFR phosphorylation and consequently activation of MAPK pathway [79]. Controversially to other reports, we found a significant higher DT in HeLa cells than in SiHa (Figure 4.3 C). However the DT of HeLa was closer to the value already described by other authors [80,81]. EGFR signaling is composed by a cascade of mediators, which culminates in the activation of transcription factors. Concerning to cell cycle, the progression from G1 to S phase is closely related to the sequential activation of cyclins and cyclin dependent kinases (CDK) [82]. An important role of MAPK activated transcription factors is to induce the production of cyclin D, which are the group of cyclins that respond to external stimuli and initiate cell cycle as a complex with CDK4/6 [52,83]. Previous studies have revealed that EGFR activation leads to the cyclin D1 upregulation and downregulation of cyclin-dependent kinase inhibitor proteins [52,55]. This corroborates our results showing higher amounts of cyclin D1 in both cell lines after EGF stimulation (Figure 4.3 E) concomitant with an accelerated GO/G1 cell cycle phase (Figure 4.3 D) and an increased cell proliferation.

Several immunohistochemical studies showed that high levels of laminin-332 expression were found in several human cancers, including uterine cervix cancer [17,23,33]. These alteration results in an increase of invasive capacity and tumor growth, as a result of proteolytic fragments of laminin-332, which have the capacity to activate proliferative signaling pathways [18]. Tran and collaborators (2008) demonstrated that, *in vivo*, globular domain 4-5 of $\alpha 3$ stimulates PI3K and MAPK pathways in carcinoma [25]. Moreover, Mizushima et al. (1996) showed that EGF strongly enhanced the expression of genes that codify laminin-332 subunits in carcinoma cell lines of stomach, bladder and vulva [84]. However, the role of EGF on laminin-332 chains expression is not clear in uterine cervix cells. The mRNA expression results showed that in basal conditions SiHa cells express significantly higher levels of all laminin-332 chains, particularly the LAMC2 chain (Figure 4.4 A). Despite this difference, both cell lines had similar responses to EGF-stimuli, as shown by mRNA expression levels of all laminin chains analyzed. LAMC2 expression was significantly upregulated after EGF supplementation (Figure 4.4 B and C). Unlike Mizushima and collaborators observations, our results only showed an upregulation of LAMC2 [84]. This findings are in agreement with other reports that demonstrated an increased expression of LAMC2 and EGFR in several cancer cases [85,86] and cancer cell lines [29,40]. In addition, it has also been shown direct interactions between the LAMC2 III domain and EGFR, resulting in downstream pathway activation [17,23]. Together, these findings suggest a positive-feedback loop associated with tumor progression, migration, and invasion by modulating signaling through EGFR. Unfortunately, we did not have the opportunity neither to evaluate EGFR expression and activation, nor the release of LAMC2 fragments that would be able to activate EGFR. However, we will be address these in future studies.

Our findings showed an *in vitro* upregulation of LAMC2 expression by EGF, which also promotes cell proliferation. Thus, putting it together with literature, a LAMC2 silencing assay was performed in order to evaluate if the levels of LAMC2 would somehow affect the role of EGF. The results of cell cycle assay showed that cell proliferation upon EGF exposure was independent of LAMC2 subunit in SiHa cell lines, but in HeLa cells the depletion of LAMC2 increased the response to EGF (Figure 4.5 A and B). There are contradictory findings about LAMC2 knockdown and its alteration in proliferation potential. In lung adenocarcinoma, either the ectopic expression of LAMC2 or knockdown did not affect

cell proliferation, whereas in anaplastic thyroid carcinoma the reduction of LAMC2 protein caused a cell cycle arrest and inhibited cell proliferation [29,32]. In contrast, our results showed a tendency of speeding up cell cycle in LAMC2 knocked down cells, as observed either by the proliferation curves and by the calculation of population DT (Figure 4.5 C, D and E). Interestingly, the delay time results of cell cycle phases, in agreement with our previous results from WT cells, showed a shortening of the first cell cycle phase, G0/G1 (Figure 4.5 F).

The capacity of tumor cells to migrate and invade through BM and ECM are key processes of metastasis [87]. The role of LAMC2 in cell migration was measured by wound healing assay (Figure 4.6) and no differences were detected between HeLa, even in EGF-treated groups (*supplementary Figure 1*). In the other hand, in SiHa cells the EGF supplementation was essential to promote directional cell migration in control groups (WT and shControl), however, the knockdown of LAMC2 leaded to a decrease in cell migration. Moreover, stimulation with EGF also was necessary to induce invasion of SiHa cells (Figure 4.7). Although, the lack of LAMC2 chain inhibited the EGF effect. Taken together, these observations suggest that LAMC2 is involved in cell migration and invasion in SiHa cells (squamous cell carcinoma). Although, there are no descriptions on the effects of LAMC2 downregulation in uterine cervix *in vitro* models, it has already been demonstrated that the silencing of LAMC2 led to a decrease in cell migration and invasion of squamous cell carcinoma cell lines from head and neck, which in part supports our findings for SiHa cells [61]. Moon and collaborators (2015) showed that secreted LAMC2 enhances cell migration and invasion, as well as enhances the metastatic potential of lung adenocarcinoma [32]. However, our results about LAMC2 silencing in HeLa, uterine cervix adenocarcinoma, pointed to the opposite direction.

In cancer microenvironment, the upregulation of extracellular proteolysis plays essential role in tumor growth, tissue remodeling, inflammation, tissue invasion, and metastasis. Several MMPs have been described as tumor microenvironment modulators, such as MMP2 and MMP9 [43]. The zymography results showed that, in control conditions, knockdown of *LAMC2* leads to a decrease in MMP2 activity. However, the EGF stimulation restores the enzyme activity in SiHa cells (Figure 4.8 A and C). These results are in agreement with other studies, where the proteolytic cleavage of LAMC2 chain was related to MMP2 activity. Moreover, EGFR pathway activation can regulate MMP2 expression [44,45]. In HeLa cells, the MMP2 activity after EGF treatment was not changed (Figure 4.8 A and C). Concerning the MMP9, our results showed an increase of enzyme activity in the HeLa cells treated with EGF, although, in the SiHa cells, this increase was only observed in shLAMC2 group (Figure 4.8 A and B). This corroborates the observation of other authors where MMP9 was activated by growth factors [88].

Concerning the expression of laminin-332 chains our results are in accordance with the literature, as all LAMA3, LAMB3 and LAMC2 are more expressed in SiHa cells (squamous cell carcinoma) than in HeLa cells (adenocarcinoma) (Figure 4.9) [42]. The immunofluorescence results for all laminin-332 chains, confirmed the downregulation of LAMC2 levels after transfection with shRNA in both cell lines (Figure 4.9 A and D). As expected, the protein depletion was not complete, once this effect is rarely observed using ShRNA technology [89]. The knockdown effect on the others laminin-332 chains led to similar results between cell lines: a decrease of LAMA3 expression (Figure 4.9 B and D), whereas the LAMB3 protein did not changed, compared to shControl group (Figure 4.9 C and D).

To understand the effect of EGF and LAMC2 levels in the expression of the others γ chains, shControl and shLAMC2 cells were cultured in the presence or absence of EGF and LAMC1 and LAMC3 mRNAs were quantified. The most interesting result concerns the regulation of LAMC1 gene expression. In SiHa cells, the LAMC2 knockdown led to a significant increase among conditions (Figure 4.10 C). Once again, under the same conditions, the LAMC2 knockdown in HeLa cells had the opposite effect in comparison to SiHa (Figure 4.10 D). In SiHa cells, despite the slight increase of LAMC3 expression in shLAMC2 under control conditions, the expression of this gene was not affected by EGF

(Figure 4.10 C and E). However, in HeLa cells, the knockdown had no effect on LAMC3 expression while the EGF supplementation leads to a decrease of its mRNA levels (Figure 4.10 F). Therefore, the following assays of EGF-mediated regulation were performed for LAMC2 as well as LAMC1. Surprisingly, an unexpected increase of LAMC2 mRNA levels in ShLAMC2-SiHa cells upon EGF stimuli was observed (Figure 4.10 A). So, a pulse chase assay was performed to clarify this result (Figure 4.11). The results showed that EGF stimuli resulted in a higher gene expression of *LAMC2*. As expected, this expression level was lower in knockdown groups, in both cell lines, demonstrating that silencing of LAMC2 was effective.

Recently, our group demonstrated that STAT3:FOXM1 pathway is upregulated and associated with uterine cervix carcinogenesis and cancer progression [6]. As STAT3 and FOXM1 can be modulated by EGF signaling pathways, we evaluated the binding levels of STAT3 and FOXM1 in *LAMC1* and *LAMC3* promoters. Our results showed a similar pattern of regulation between LAMC1 and LAMC2 promoter regions (Figure 4.13). In SiHa cells, EGF regulated significantly both promoter regions through FOXM1 binding, especially *LAMC2* promoter. In HeLa cells, the EGF supplementation led to a slightly increase (not significant) of FOXM1 and STAT3 to LAMC2 promoter and to a significant increase of STAT3 binding to LAMC1 promoter.

The results of immunofluorescence, in order to confirm the regulation of EGF stimulation, showed an increase of LAMC2 protein levels under EGF supplementation in both cell lines (Figure 4.12 A and C). In SiHa this increase can be related to the increase of FOXM1 under the same conditions. Its binding can justify the 1.4-fold increase of LAMC2. In another hand, the LAMC2 increase in HeLa is possibly related to STAT3 binding on *LAMC2* promoter region. These results are in agreement with the previous detected mRNA levels of these protein under control conditions or under EGF stimulation. Regarding LAMC1, the results of FOXM1 and STAT3 regulation are contradictory, since we found that the expression of this protein is not altered by EGF stimulus in SiHa WT cells, as much as it was not possible to detect by immunofluorescence (Figure 4.12 B and D). In HeLa WT cells, the immune staining of LAMC1 showed an upregulation of this protein after EGF supplementation, and it can be a clue on the action of LAMC1 in adenocarcinoma phenotype. This protein increase could be due to the increase of STAT3 binding to *LAMC1* promoter. The role of LAMC1 in SiHa cells must be also relevant since this cell line expresses higher levels of LAMC1 than HeLa, but in SiHa LAMC1 expression was not altered by EGF. Anyway, western blotting analysis will be performed in order to assess more accurately the quantification of LAMC1 and LAMC2.

After realizing that LAMC1 was increased with the knockdown of LAMC2, we wanted to understand further about the relevance of LAMC1 in uterine cervix cancer. The results of LAMC1 expression in cancer samples in comparison to normal uterine cervix tissues were contradictory between the analyzed databases. However, when data were joined, the result reveals a significantly higher expression in tumor samples (Figure 4.14 C). According to the literature, there are findings showing the increase of LAMC1 expression in tumor tissues comparatively to normal tissues, in colorectal cancer [90]. Moreover, in endometrial carcinoma LAMC1 expression is associated with high grade and tumor progression [91]. We believe LAMC1 is an interesting gene to address in further studies and we aim to clarify its role in uterine cervix cancer, mainly in adenocarcinomas.

5.1 Main conclusions

The central aim of this thesis was to clarify the EGF mediated role of LAMC2 of laminin-332 in the progression of uterine cervix carcinomas, using an in vitro model of squamous cell carcinoma (SiHa) and adenocarcinoma (HeLa).

In the first approach were confirmed in human cancer databases that LAMC2 is upregulated in several cancer types and its expression also is increased in uterine cervix cancer. The cell cycle analyses

revealed that both cell lines are EGF-responsive, as they proliferate more upon EGF stimulus, so we confirmed that EGF is a suitable growth factor to stimulate uterine cervix cancer cells. Our results showed that EGF stimulation results in a shortened duration of G0/G1 cell cycle phase and an increased percentage of cells in S+G2/M phases, concomitant with increased cyclin D1 levels. It was also found that EGF regulates the expression of LAMC2 in both cell lines (squamous cell carcinoma and adenocarcinoma). We have good indications that FOXM1 (in SiHa) and STAT3 (in HeLa) are pivotal in the regulation of LAMC2 by EGF. LAMC2 knockdown showed that EGF stimulates proliferation independently of LAMC2 in SiHa cells, but interestingly in HeLa the pro-proliferative effect of EGF is more efficient in the absence of LAMC2. Moreover, the LAMC2 silencing suppresses SiHa ability to migrate and invade. The activity of MMP2 and MMP9 is regulated by EGF and the level of activity of MMP9 is related to LAMC2 levels, in both cell lines. The MMP2 activity is affected by LAMC2 levels in opposite ways in each cell line: LAMC2 knockdown decreases MMP2 activity in SiHa and increases is HeLa. So, it seems that the malignant phenotype of squamous cell carcinoma relies more on LAMC2 than the malignant phenotype of adenocarcinoma.

The LAMC2 knockdown induces the transcription of LAMC1 in SiHa at mRNA levels. Under control conditions, the same result was observed for *LAMC3*, although it had a lower significance which disappeared with EGF treatment. In wild type (WT) cell lines upon EGF stimulus the protein levels of LAMC1 increases in HeLa not in SiHa. Despite the need of confirming this result by western blotting, it seems that this laminin gene can play a role in the absence of LAMC2 in squamous cell carcinoma (SiHa) and under the EGF influence it can confer a mild malignant phenotype in adenocarcinoma (HeLa--low invasive profile). Again, it seems that EGF also plays a role in LAMC1 expression in SiHa and HeLa cells and it is necessary to depict the role of FOXM1 and STAT3. By analyzing human cancer databases, we confirmed that LAMC1 association with cancer is controversial as stated in the literature, even in uterine cervix cancer. Unfortunately, in databases the histological type of uterine cervix cancer is not identified, so we cannot analyze the levels of LAMC1 within squamous cell carcinoma and adenocarcinoma groups.

We believe this thesis gives relevant insights on the role of regulatory dynamics of LAMC2 by EGF that accounts for the uterine cervix squamous cell carcinoma aggressive phenotype. We also pointed LAMC1 as a putative key element in uterine cervix cancer progression. New perspectives of research can be designed from our results so that a better understanding of the molecular mechanisms underlying different routes of progression of different cancer histological types, despite they develop in the same organ.

5.2 Future perspectives

As future perspectives, for better understanding of the role of LAMC2 and LAMC1 in uterine cervix cancer, it would be important to:

- Evaluate the expression and the activation status of EGFR;
- Consolidate the LAMC2 knockdown experiments by using lentiviral vectors;
- Evaluate the LAMC2 and LAMC1 expression by western blotting, under control and EGF conditions;
- Analyze the MAPK signaling pathways activation status;
- Perform FOXM1 and STAT3 knock down experiments, and evaluate the expression of LAMC2 and LAMC1 by immunohistochemistry in patients samples;

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Appendices

Solutions prepared for the experimental work:

10X PBS (pH 7.4-7.6)
For 1L: 80g NaCl (1.37M) (106404, Merck) 2g KH_2PO_4 (14.7mM) (104873, Merck) 11.1g Na_2HPO_4 (78.1mM) (S-0876, Sigma) 2g KCl (26.8mM) (104936, Merck) ddH_2O to 1L

PBS 0.2% (w/v) BSA
0.4g BSA (A9647, Sigma) 200 mL 1X PBS

50 µg/mL Propidium Iodide (PI) solution – Cell cycle assay
For 50 mL: 1 mL of 2.5 mg/mL PI solution (P4170, Sigma) (prepared in 1X PBS) 49 mL 1X PBS 0.1 mg/mL RNase A (Easy spin kit, Citomed) 0.05% Triton X-100 (T8787, Sigma)

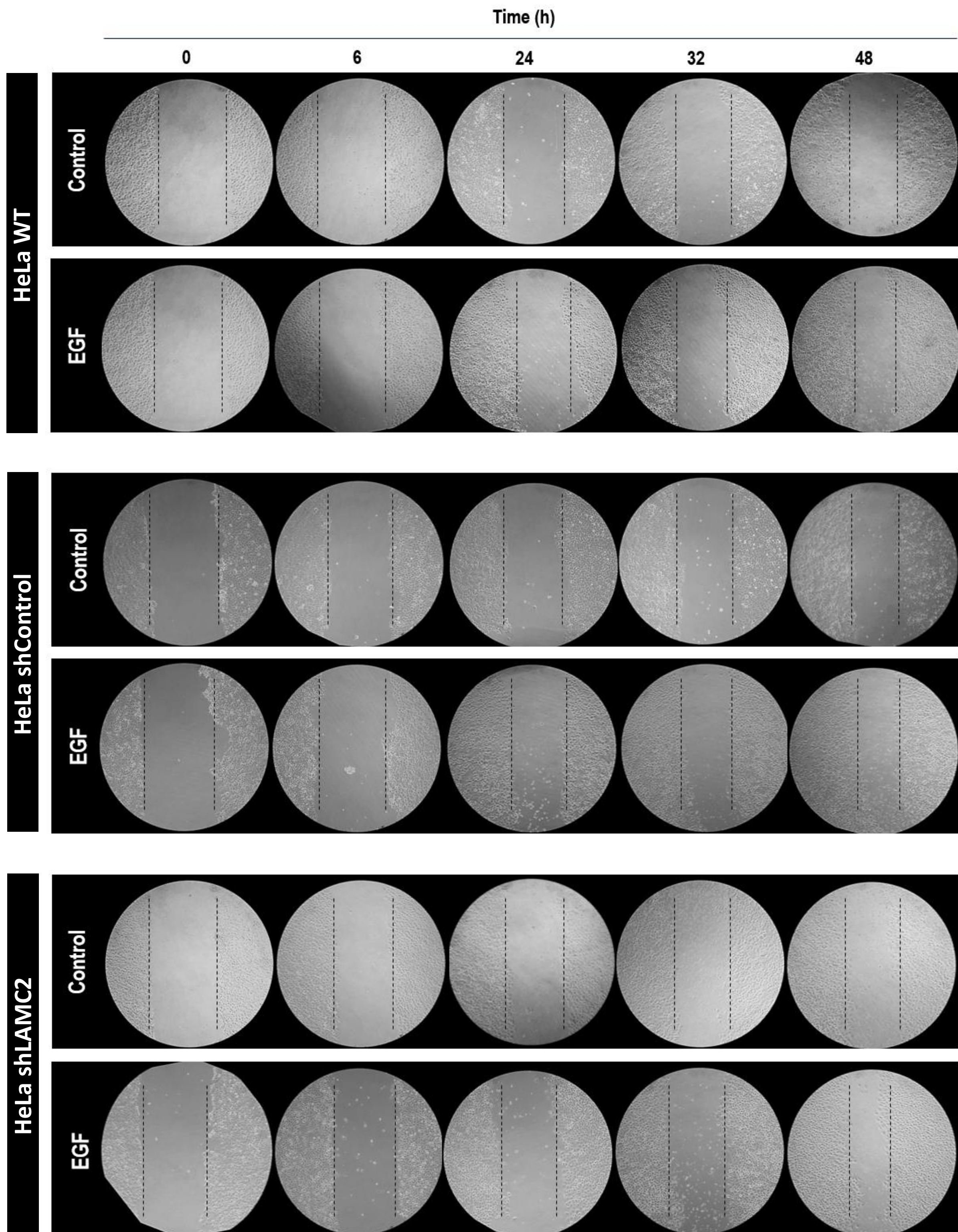
Zymography solutions	
Running gel (10% polyacrilamide gel with gelatin) For 10 ml: 3.33 ml 30% (w/v) acrylamide 2.5 ml 1.5 M Tris, pH 8.8 3 ml distilled water 1 ml 1.2% (w/v) gelatin 50 µl 20% (/w/v) SDS (sodium dodecyl sulfate) 33.3 µl 10% (w/v) APS (ammonium persulfate) 6.7 µl TEMED (Tetramethylethylenediamine)	**Stacking gel** For 3 ml: 500 µl 30% acrylamide 380 µl 1.0 M Tris, pH 6.8 2.1 ml distilled water 30 µl 10% (w/v) SDS (sodium dodecyl sulfate) 30 µl 10% (w/v) APS (ammonium persulfate) 3 µl TEMED (Tetramethylethylenediamine)
Low salt collagenase buffer 10 X For 1L: 60.6 g Tris base 117 g sodium chloride 5.5 g calcium chloride Distilled water up to 1 L pH 7.6	**Collagenase buffer 1X** For 1L: 100 ml stock solution 900 ml distilled water 670 µl 30% (w/v) Brij
Destain solution For 1L: 100 ml glacial acetic acid 300 ml methanol 600 ml distilled water	
Coomassie brilliant blue stock solution For 500 ml: 250 ml methanol 0.25 g Coommassie brilliant blue 200 ml distilled water 50 ml acetic acid	**Coomassie working dilution** For 500 ml: 150 ml coomassie brilliant blue stock solution 350 ml destain solution

Reference:

Sambrook J, Russel DW (2001) Molecular cloning: a laboratory manual, 3rd edn. New York: Cold Spring Harbor Laboratory Press.

***Supplementary Table 1* – Primers sequences used during the experimental work.**

Primer	Forward (5´-3´)	Reverse (5´-3’)
LAMA3	CACTCGGCGGTATTATTACAC	CTGGCATTCACACCGAAACAG
LAMB3	CCGAGTGGCAGATGAAATGG	CAGAGAGACAGGGTTCACATC
LAMC2	CAAGACCAGAGACCTGCTAC	CTGATCGACACCTATCACAGC
LAMC1	CTGCACCTGGGAAAAGCTTTG	GTGTTCTCACAGGAACCACTG
LAMC3	CCTAGGGAAGGCTTATGAGATC	CTGTAGAACTGGTAGGGCTC
CCND1	CACGCGCAGACCTTCGTT	CATGGAGGGCGGATTG
LAMC2 promoter	CCTCCTTATTCACAGGTGAGTC	CTTCTACCTGACTCAGTCCTG
LAMC1 promoter	GTCCTATATGCCCACGTTTGTC	GGGAGAATTAAGTTGTGGGGAC
HPRT	TGACACTGGCAAAACAATGCA	GGTCGTTTTTCACCAGCAAGCT



***Supplementary Figure 1 - LAMC2 does not affect HeLa cells migration*.** Comparison between WT SiHa cells, shControl and shLAMC2, at 0, 6, 24, 32 and 48 hours, with and without EGF. The result shows that in HeLa cells, both EGF treatment and knockdown of LAMC2 had no effect on migration. Phase microscopy (original magnification: 200x).