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Jörg Reichrath
Sandra Reichrath *Editors*

Notch Signaling in Embryology and Cancer

Notch Signaling in Cancer

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We dedicate this book to our sons, Benjamin and Niklas, the absolute joy of our lives

Preface

Notch and Cancer: Lessons Learned from Failure – or on the Long and Winding Road from John S. Dexter's mutant Fruit Flies to Synthetic Notch Receptors, and beyond

This book was motivated by the desire we and others have to further the evolution of the many fascinating facets of Notch's role for biology and medicine. To stimulate the interest of potential readers, this preface has several purposes, which include providing some brief information (a) concerning its content with a focus on potential future developments, (b) how the book came about, and (c) acknowledging the long list of authors and other individuals who helped move forward this project. The success of the first edition of *Notch Signaling in Embryology and Cancer*, which was published by Landes and Springer in 2012 in the prestigious series Advances in Experimental Medicine and Biology (Reichrath and Reichrath 2012), clearly indicated that this text met an important need in this exciting field. It was a benchmark in its field, fulfilling the need to provide a broad audience (ranging from medical students to basic scientists, physicians and all other health care professionals) with up-to-date information in a comprehensive, highly readable format, with individual chapters written by highly respected experts. We were aware from the beginning that undertaking the first edition would include a commitment to producing a second one. The enormous advances that have been made in recent years on this topic, moving forward at a dizzying pace, now clearly justify the need for a second edition. We decided to publish this updated and extended new edition as three separate volumes, featuring chapters written by both new and returning authors, to add to the content of the first edition. Each volume contains a separate table of contents and full index to help the reader find specific information. In the different volumes of *Notch Signaling in Embryology and Cancer*, leading experts in the field present a comprehensive, highly readable overview on selected aspects of three important topics related to Notch signaling, namely the underlying molecular mechanisms that mediate its biological effects (Volume I) (Reichrath and Reichrath 2020a) (adding to the recent important book publication on this topic from Tilman Borggrefe and Benedetto Daniele Giaimo; Borggrefe and Giaimo 2018), its role in embryogenesis (Volume II) (Reichrath and Reichrath 2020b), and last but not least its relevance for pathogenesis, progression, and therapy of

cancer (Volume III) (Reichrath and Reichrath 2020c). This third volume of *Notch Signaling in Embryology and Cancer* summarizes the fascinating role of this pathway, which first developed during evolution in metazoans and was first discovered in the fruit fly *drosophila melanogaster*, for cancer (Reichrath and Reichrath 2020c). When the American scientist John S. Dexter published in 1914 the characteristic notched-wing phenotype (a nick or notch in the wingtip that earned the mutant gene the name Notch) that he had recognized in mutant fruit flies (*drosophila melanogaster*), he couldn't have expected the tremendous impact that this finding would later have for cancer and many other fields in biology and medicine. This thoughtful observation made in his research laboratory at Olivet College (Olivet, Michigan, USA) opened many fascinating avenues for a better understanding of carcinogenesis and for the development of promising new anti-cancer therapeutics that target Notch signaling pharmacologically. During the last decades, a great deal of impressive scientific progress has demonstrated that Notch signaling represents a master pathway involved in carcinogenesis and cancer progression, with many types of cancer having been found to contain Notch mutations. On a first look, the Notch pathway, which governs in oncology so many key cell fate decisions and other cellular core processes, seems phantasmally simple, because a direct link between an extracellular signal and transcriptional output without the requirement of an extended chain of protein intermediaries (as needed by so many other signaling pathways) represents one of its key features (Reichrath and Reichrath 2020a, b). However, on a second, closer look, this obvious simplicity hides remarkable complexity and, consistent with its central role in many aspects of development, adult tissue homeostasis, and cancer, it can be recognized that Notch signaling depends on an extensive collection of mechanisms that it employs alongside of its core transcriptional machinery. It has to be noted that many early attempts to target Notch signaling for cancer treatment represented major setbacks, since they did not lead as expected to good clinical response rates. Notch antagonistic antibodies and gamma secretase inhibitors (GSI) may serve here as examples, showing promising results in preclinical studies but disappointing results in clinical trials. However, our understanding of the molecular biology of Notch signaling has now opened exciting perspectives to overcome these obstacles. Giulia Monticone and Lucio Miele discuss elsewhere in this book (Monticone and Miele 2020), in their direction-defining contribution, which reflects the outstanding expertise of the authors, current and new Notch-targeting therapies with their exciting promises and challenges. As these authors explain, targeting Notch allows to virtually modulate any aspect of cancer. However, this means that Notch-targeting must be highly specific toward the desired target and it seems that we at present still have not unraveled all the secrets and potentials of this pathway (Monticone and Miele 2020). In this context, Notch pleiotropic nature seems to be both the advantage and the challenge of Notch-targeted therapies (Monticone and Miele 2020). The accumulated knowledge about the molecular biology of Notch signaling in individual types of cancer, for example, whether Notch muta-

tions detected in cancer are assumed to be loss or gain of function depending on whether Notch is tumor suppressor or oncogenic, respectively, provides chances for improvements, for it may be possible to identify responder patients depending on molecular signatures detected in tumors (Monticone and Miele 2020). It is now becoming evident that while the first Notch-targeted therapies were designed to inhibit Notch, in certain situations Notch signaling should be promoted instead of inhibited (Monticone and Miele 2020). Recent investigations on Notch regulation have revealed many alternative ways in which Notch can be activated or inhibited, which involve the extensive collection of mechanisms that Notch employs alongside of its core transcriptional machinery. Other mechanisms that can be used as therapeutical targets include the ubiquitination of Notch mediated by different ubiquitin ligases that orchestrate the degradation and the ligand-independent activation of Notch. Moreover, Notch's roles for microenvironment and for metabolism reprogramming (now considered as a major hallmark of cancer, through which cancer cells can adapt and survive different environmental changes, develop resistance to treatments, and modulate anti-tumor immunity) of many tumor types may represent targets for anti-cancer treatment (Monticone and Miele 2020). Understanding whether Notch function is pro- or anti-tumoral is essential, especially because Notch is differentially expressed in subsets of cells within the tumor and its microenvironment including tumor-infiltrating immune cells (Monticone and Miele 2020). Notch is heavily involved in shaping the immune system in physiological conditions and the pro-tumoral immune microenvironment in cancer (Monticone and Miele 2020), providing a strong rational for the evaluation of Notch-targeting strategies as immunomodulators (Monticone and Miele 2020). The promises associated with the many fascinating facets of targeting Notch signaling for cancer treatment include the generation of synthetic Notch receptors, called synNotches, which have customizable extracellular and intracellular domains linked by the transmembrane domain of Notch, thus allowing customizable extra to intracellular signaling. In conclusion, a more rational use of Notch-targeting therapeutics should be highly specific, taking into account many aspects, including the tumor type, patient responders, Notch alterations, "off targets," and potential combinatorial treatments. Understanding the precise mechanism by which Notch is modulated in different sets of cells within the tumor or its microenvironment including immune cells will be crucial to predict whether Notch-targeting therapies will be effective and to identify new druggable targets (Monticone and Miele 2020). Therefore, a patient-based, mechanistic-based use of Notch-targeting therapies is urgently needed. The individual chapters of this book give an up-to-date overview on selected aspects of our present understanding of Notch's role in cancer. We have enjoyed very much the task of bringing this second edition to you. We are convinced that it will be as successful as the previous edition. We are indebted to our many authors and are very grateful for their willingness to contribute to this book. We would also like to express our thanks to Murugesan Tamilselvan, Anthony Dunlap, Larissa Albright, and all the

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Enjoy the reading!

Homburg, Saarland, Germany

Sandra Reichrath
Jörg Reichrath

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1 Notch Signaling in Prevention And Therapy: Fighting Cancer with a Two-Sided Sword

Jörg Reichrath and Sandra Reichrath

Abstract

The evolutionary conserved Notch pathway that first developed in metazoans and that was first discovered in fruit flies (*Drosophila melanogaster*) governs fundamental cell fate decisions and many other cellular key processes not only in embryonic development but also during initiation, promotion, and progression of cancer. On a first look, the Notch pathway appears remarkably simple, with its key feature representing a direct connection between an extracellular signal and transcriptional output without the need of a long chain of protein intermediaries as known from many other signaling pathways. However, on a second, closer look, this obvious simplicity exerts surprising complexity. There is no doubt that the enormous scientific progress in unraveling the functional mechanisms that underlie this complexity has recently greatly increased our knowledge about the role of Notch signaling for pathogenesis and progression of many types of cancer. Moreover, these new scientific findings have shown promise in opening new avenues for cancer prevention and therapy, although this goal is still challenging. Vol. III of the second edition of the book *Notch Signaling in Embryology and Cancer,* entitled *Notch Signaling in Cancer*, summarizes important recent developments in this fast-moving and fascinating field. Here, we give an introduction to this book and a short summary of the individual chapters that are written by leading scientists, covering the latest developments in this intriguing research area.

Keywords

Angiogenesis · Cancer · Cancer stem cells · Cancer treatment · Notch · Non-melanoma skin cancer · Notch signaling · Notch pathway · Skin cancer · Tumor angiogenesis

Abbreviations

BCC	Basal cell carcinoma
CSC	Cancer stem cell
Dll	Delta-like
ESCC	Esophageal squamous cell carcinoma
Hes	Hairy and enhancer of split

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HNSCCs	Head and neck squamous cell cancers
HPV	Human papillomavirus
Hrt	Hes-related transcription factor
JAG	Jagged
NID	Notch intracellular domain
NMSC	Non-melanoma skin cancer
SCC	Squamous cell carcinoma

Evolutionary conserved Notch signaling that first developed in metazoans (Gazave et al. 2009; Richards and Degnan 2009) and that was first discovered in the fruit fly *Drosophila melanogaster* represents one of the most fascinating pathways that govern both embryonic development and adult tissue homeostasis and an essential element of the defense line against cancer. Notably, the fascinating tale that earned the gene the name *Notch* began over a century ago, when the American scientist John S. Dexter discovered at Olivet College (Olivet, Michigan, USA) the typical notched-wing phenotype (a nick or notch in the wingtip) in his stock of mutant fruit flies *Drosophila melanogaster* (Dexter 2014; Reichrath and Reichrath 2020a, b). The alleles responsible for this phenotype were identified 3 years later at Columbia University (New York City, New York, USA) by another American scientist, Thomas Hunt Morgan (1866–1945) (Morgan 1917; Reichrath and Reichrath 2020a, b). In the following years, many additional alleles were identified that were associated with the Notch phenotype (Morgan 1928; Reichrath and Reichrath 2020a, b). In subsequent decades, notwithstanding the extensive research on the *Notch* locus, researchers struggled to identify the function for the *Notch* gene due to the lethality early in embryogenesis and the broad variety of phenotypic consequences of Notch mutants (Reichrath and Reichrath 2020a, b). Despite these challenges, the observations of John S. Dexter, Thomas Hunt Morgan, and others were finally confirmed by cloning and sequencing of the mutant *Notch* locus in the research laboratories of Spyros Artavanis-Tsakonas and Michael W. Young, more than half a century later (Wharton et al. 1985; Kidd et al. 1986). A huge mountain of new scientific evidence, which has been constantly growing during the last decades, has now convincingly shown that the Notch pathway governs, from sponges, roundworms, *Drosophila melanogaster*, and mice to humans, many key cell fate decisions and other core processes that are of high importance both for embryogenesis and in adult tissues (Andersson et al. 2011). Moreover, it has now been demonstrated that Notch signaling represents in humans an essential element of the defense line against cancer.

In 2012, when the first edition of *Notch Signaling in Embryology and Cancer* was published by Landes and Springer in the prestigious series *Advances in Experimental Medicine and Biology*, it was the benchmark on this topic, providing a broad audience (ranging from medical students to basic scientists, physicians, and all other healthcare professionals) with up-to-date information in a comprehensive, highly readable format. Since that time, a huge mountain of new scientific findings has been built up that underlines the many facets and the high biological/clinical relevance of Notch signaling for health and many diseases, including various types of cancer (Reichrath and Reichrath 2020a, b). Therefore, we decided that it is now the right time to publish an updated and extended version. The second edition of this book has been expanded substantially to cover all aspects of this fast-growing field and has been divided into three separate volumes to include additional chapters (Reichrath and Reichrath 2020a, b). In this new edition, leading scientists provide a comprehensive, highly readable overview on molecular mechanisms of Notch signaling (Volume I), Notch's role in embryonic development (Volume II), and, last but not least, its relevance for cancer (Volume III) (Reichrath and Reichrath 2020a, b).

This third volume gives an overview on Notch's role for selected types of cancer. As outlined previously, it must be emphasized that the Notch pathway seems delusorily simple, with one of its key features being a direct link between an extracellular signal and transcriptional output without the requirement for an extended chain of

protein intermediaries as needed by so many other signaling pathways (Hunter and Giniger 2020; Reichrath and Reichrath 2020a, b). However, this apparent simplicity hides remarkable complexity, and, consistent with its important role in many aspects of development, it has to be noted that Notch signaling has an extensive collection of mechanisms that it exerts alongside of its core transcriptional machinery (Hunter and Giniger 2020; Reichrath and Reichrath 2020a, b). In many biological processes, including morphological events during pathogenesis and progression of cancer, Notch-mediated coordination of the activity of gene expression with regulation of cell morphology is of high importance (Hunter and Giniger 2020; Reichrath and Reichrath 2020a, b). Fortunately, the generation and investigation of knockout mice and other animal models have in recent years resulted in a huge mountain of new informations concerning Notch gene function, allowing to dissect the role of specific Notch components in human development and disease. This volume is intended to provide both basic scientists and clinicians who seek today's clearest understanding of the molecular mechanisms that mediate Notch signaling with an authoritative day-to-day source.

In the first chapter, Benedetto Daniele Giaimo, Ellen Kolb, Rhett A. Kovall, and Tilman Borggrefe convincingly demonstrate the importance of the transcription factor RBP-J as a molecular switch in regulating the Notch response (Benedetto Daniele Giaimo et al. 2020). As they explain, the Notch signal transduction cascade requires cell-to-cell contact and results in the proteolytic processing of the Notch receptor and subsequent assembly of a transcriptional coactivator complex containing the Notch intracellular domain (NID) and transcription factor RBP-J. In the absence of a *Notch* signal, RBP-J remains at Notch target genes and dampens transcriptional output. Like in other signaling pathways, RBP-J is able to switch from activation to repression by associating with corepressor complexes containing several chromatin-modifying enzymes. In their chapter, Giaimo et al. focus on the recent advances concerning RBP-J corepressor functions, especially in regard to chromatin regulation. The authors put this into the context of one of the best-studied model systems for Notch, blood cell development. They elaborate that alterations in the RBP-J corepressor functions can contribute to the development of leukemia, especially in the case of acute myeloid leukemia (AML). The versatile role of transcription factor RBP-J in regulating pivotal target genes like *c-MYC* and *HES1* may contribute to the better understanding of the development of leukemia.

In the following chapter, Tobias Reiff, Miriam Baeumers, Christine Tibbe, and Thomas Klein provide a review on the role of the tumor suppressor lethal (2) giant discs (Lgd)/CC2D1, Notch signaling, and cancer (Reiff et al. 2020). They state that the endosomal pathway plays a pivotal role upon signal transduction in the Notch pathway and that recent work on *lethal (2) giant discs* (*lgd*) points to an additional critical role in avoiding uncontrolled ligand-independent signaling during trafficking of the Notch receptor through the endosomal pathway to the lysosome for degradation. In their chapter, the authors line out the fascinating journey of Notch through the endosomal system and present an overview of the current knowledge about Lgd and its mammalian orthologs Lgd1/CC2D1b and Lgd2/CC2D1a. They further discuss how Notch is activated in the absence of *lgd* function in *Drosophila* and ask whether there is evidence that a similar ligand-independent activation of the Notch pathway can also happen in mammals if the orthologs are inactivated.

In the next chapter, Violeta Jonusiene and Ausra Sasnauskiene summarize the relevance of Notch for endometrial cancer (Jonusiene and Sasnauskiene 2020). They explain that human endometrium is a unique, highly dynamic tissue that undergoes cyclic changes of cell proliferation, differentiation, and death. Endometrial cancer is the most common malignancy among women in developed countries. Importantly, the incidence of endometrial cancer is rising in high-income countries. Currently histological classification is used for subtyping of endometrial cancer, while ongoing research is evaluating markers for more accurate molecular classification. As the authors point out, accumulating evi-

dence links aberrant Notch signaling with diseases such as hyperplasia and endometrial cancer. This chapter summarizes the current state of Notch signaling investigations in the endometrium, endometriosis, and endometrial cancer.

In the following chapter, Yong Li, Yahui Li, and Xiaoxin Chen review our scientific knowledge of Notch's role in esophageal squamous cell carcinoma (ESCC) (Li et al. 2020). The authors explain that ESCC is a deadly disease that requires extensive research on its mechanisms, prevention, and therapy. Recent studies have shown that *NOTCH* mutations are commonly seen in human ESCC. This chapter summarizes our current understanding of the Notch pathway in normal esophagus and in ESCC. The authors explain that in normal esophagus, Notch pathway regulates the development of esophageal squamous epithelium, in particular, squamous differentiation. Exposure to extrinsic and intrinsic factors, such as gastroesophageal reflux, alcohol drinking, and inflammation, downregulates the Notch pathway and thus inhibits squamous differentiation of esophageal squamous epithelial cells. In ESCC, Notch plays a dual role as both a tumor suppressor pathway and an oncogenic pathway. In summary, further studies are warranted to develop Notch activators for the prevention of ESCC and Notch inhibitors for targeted therapy of a subset of ESCC with activated Notch pathway.

In the next chapter, Kazunori Kawaguchi and Shuichi Kaneko report on the relevance of Notch signaling for liver cancer (Kawaguchi and Kaneko 2020). They point out that interactions between liver cells are closely regulated by Notch signaling. Notch signaling has been reported clinically related to bile duct hypogenesis in Alagille syndrome, which is caused by mutations in the Jagged1 gene. Notch activation and hepatocarcinogenesis are closely associated since cancer signaling is affected by the development of liver cells and cancer stem cells. Gene expression and genomic analysis using a microarray revealed that abnormalities in Notch-related genes were associated with the aggressiveness of liver cancer. This pattern was also accompanied with α-fetoprotein- and EpCAM-expressing phenotypes in vitro, in vivo, and in clinical tissues. Hepatitis B or C virus chronic infection or alcohol- or steatosis-related liver fibrosis induces liver cancer. Previous reports demonstrated that HBx, a hepatitis B virus protein, was associated with Jagged1 expression. The authors report their finding that the Jagged1 and Notch1 signaling pathways were closely associated with the transcription of covalently closed circular hepatitis B virus DNA, which regulated cAMP response element-binding protein, thereby affecting Notch1 regulation by the E3 ubiquitin ligase ITCH. This viral pathogenesis in hepatocytes induces liver cancer. The authors conclude that Notch signaling exerts various actions and is a clinical signature associated with hepatocarcinogenesis and liver context-related developmental function.

In the next contribution, Cristina Porcheri and Thimios A. Mitsiadis report on Notch's role in head and neck cancer (Porcheri and Mitsiadis 2020). Head and neck cancer is a group of neoplastic diseases affecting the facial, oral, and neck region. It is one of the most common cancers worldwide with an aggressive, invasive evolution in the late stages of malignancy. Due to the heterogeneity of the tissues affected, it is particularly challenging to study the molecular mechanisms at the basis of these tumors, and to date we are still lacking accurate targets for prevention and therapy. The authors explain that Notch signaling is involved in a variety of tumorigenic mechanisms, such as regulation of the tumor microenvironment, cell-to-cell communication, and metabolic homeostasis. Moreover, they provide an up-to-date review of the role of Notch in head and neck cancer and draw parallels with other types of solid tumors where the Notch pathway plays a crucial role in emergence, maintenance, and progression of the disease. Additionally, the authors give a perspective view on the importance of the pathway in neoplastic development in order to define future lines of research and novel therapeutic approaches.

In the following chapter, Trianth Das, Rong Zhong, and Michael T. Spiotto explain the rele-

vance of Notch signaling for human papillomavirus-associated oral tumorigenesis (Das et al. 2020). They point out that the Notch pathway is critical for the development of many cell types including the squamous epithelium lining of cutaneous and mucosal surfaces. In genetically engineered mouse models, Notch1 acts as one of the first steps to commit basal keratinocytes to terminally differentiate. Similarly, in human head and neck squamous cell cancers (HNSCCs), Notch1 is often lost consistent with its essential tumor-suppressive role for initiating keratinocyte differentiation. However, constitutive Notch1 activity in the epithelium results in expansion of the spinous keratinocyte layers and impaired terminal differentiation which is consistent with the role of Notch1 as an oncogene in other cancers, especially T-cell acute lymphoblastic leukemia. The authors also report their previous observation that Notch1 plays a dual role as both a tumor suppressor and oncogene depending on the mutational context of the tumor. Namely, gain or loss or Notch1 activity promoted the development of human papillomavirus (HPV)-associated cancers. The additional HPV oncogenes likely disrupted the tumor-suppressive activities of Notch and enable the oncogenic pathways activated by Notch to promote tumor growth. In this review, the authors detail the role of Notch pathway in head and neck cancers with a focus on HPV-associated cancers.

In their contribution, Sandra and Jörg Reichrath summarize the impact of Notch signaling for carcinogenesis and progression of non-melanoma skin cancer (Reichrath and Reichrath 2020c). They explain that, since many decades, non-melanoma skin cancer (NMSC) is the most common malignancy worldwide. Basal cell carcinomas (BCC) and squamous cell carcinomas (SCC) are the major types of NMSC, representing appr. 70% and 25% of these neoplasias, respectively. Because of their continuously rising incidence rates, NMSCs represent a constantly increasing global challenge for healthcare, although they are in most cases nonlethal and curable (e.g., by surgery). The authors elaborate that, while at present, carcinogenesis of NMSC is still not fully understood, the relevance of genetic and molecular alterations in several pathways, including evolutionary highly conserved Notch signaling, has now been shown convincingly. Choosing NMSC as a model, the authors give in this review a brief overview on the interaction of Notch signaling with important oncogenic and tumor suppressor pathways and on its role for several hallmarks of carcinogenesis and cancer progression, including the regulation of cancer stem cells (CSCs), tumor angiogenesis, and senescence.

In the next contribution, Rachael Guenter, Zeelu Patel, and Herbert Chen summarize the role of Notch signaling in thyroid cancer (Guenter et al. 2020). They explain that thyroid cancer is the most common malignancy of the endocrine system with a steadily rising incidence. The term thyroid cancer encompasses a spectrum of subtypes, namely, papillary thyroid cancer, follicular thyroid cancer, anaplastic thyroid cancer, and medullary thyroid cancer. Each subtype differs histopathologically and in degrees of cellular differentiation, which may be in part due to signaling of the Notch pathway. The Notch pathway's role in cancer biology is controversial, as it has been shown to play both an oncogenic and tumor-suppressive role in many different types of cancer. This discordance holds true for each subtype of thyroid cancer, indicating that Notch signaling is likely cell type and context dependent. The authors explain that, whether oncogenic or not, Notch signaling has proven to be significantly involved in the tumorigenesis of thyroid cancer and has thus earned interest as a therapeutic target. The authors conclude that advancement in the understanding of Notch signaling in thyroid cancer holds great promise for the development of novel treatment strategies to benefit patients.

In the following chapter, Zacharias Fasoulakis, George Daskalakis, Marianna Theodora, Panos Antsaklis, Michael Sindos, Michail Diakosavvas, Kyveli Angelou, Dimitrios Loutradis, and Emmanuel N Kontomanolis elaborate on the relevance of Notch signaling in cancer progression (Fasoulakis et al. 2020). As they point out, the Notch signaling pathway controls cell prolifera-

tion, fate, differentiation, and cell death, by short-range signaling between nearby cells that come in contact. Fibroblasts, representing an essential for tumor growth component of stroma, have also been shown to be affected by Notch regulation. Notch gene mutations have been identified in a number of human tumors revealing information on the progression of specific cancer types, such as ovarian cancer and melanoma, immune-associated tumors such as myeloid neoplasms, but especially lymphocytic leukemia. The authors further explain that activation of Notch can be either oncogenic or it may contain growth-suppressive functions, acting as a tumor suppressor in other hematopoietic cells, hepatocytes, and skin and pancreatic epithelium.

In the next contribution, Qiang Shen and Michael Reedijk elaborate on the role of Notch signaling for the breast cancer microenvironment (Shen and Reedijk 2020). They explain that Notch promotes breast cancer progression through tumor-initiating cell maintenance, tumor cell fate specification, proliferation, survival, and motility. In addition, Notch is recognized as a decisive mechanism in regulating various juxtacrine and paracrine communications in the tumor microenvironment (TME). In this chapter, we review recent studies on stress-mediated Notch activation within the TME and sequelae such as angiogenesis, extracellular matrix remodeling, changes in the innate and adaptive immunophenotype, and therapeutic perspectives.

Last but not least, Giulia Monticone and Lucio Miele present a journey from notching phenotypes to cancer immunotherapy (Monticone and Miele 2020). The authors point out that Notch is a remarkable evolutionary conserved pathway, which has fascinated and engaged the work of investigators in an uncountable number of biological fields, from development of metazoans to immunotherapy for cancer. Nowadays Notch is the protagonist of some of the most cutting-edge fields including immunotherapy and synthetic biology. In their chapter, Monticone and Miele provide a comprehensive overview of the Notch field, with particular focus on the newest mechanistic and therapeutic advances and the future challenges of this constantly evolving field.

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2 Transcription Factor RBPJ as a Molecular Switch in Regulating the Notch Response

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Abstract

The Notch signal transduction cascade requires cell-to-cell contact and results in the proteolytic processing of the Notch receptor and subsequent assembly of a transcriptional coactivator complex containing the Notch intracellular domain (NICD) and transcription factor RBPJ. In the absence of a Notch signal, RBPJ remains at Notch target genes and dampens transcriptional output. Like in other signaling pathways, RBPJ is able to switch from activation to repression by associating with corepressor complexes containing several chromatin-modifying enzymes. Here, we focus on the recent advances concerning RBPJ-corepressor functions, especially in regard to chromatin regulation. We put this into the context of one of the best-studied model systems for Notch, blood cell development. Alterations in the RBPJ-corepressor functions can contribute to the development of leukemia, especially in the case of acute myeloid leukemia (AML). The versatile role of transcription factor RBPJ in regulating pivotal target genes like *c-MYC* and *HES1* may contribute to the better understanding of the development of leukemia.

Keywords

Notch · SHARP · KyoT2/FHL1C · L3MBTL3 · H2A.Z · p400 · Tip60 · AML1/ETO · Leukemia

Abbreviations

ADAM	A disintegrin and metalloproteinase
AE	AML1/ETO
AE9a	AML1/ETO 9a
AEtr	AML1/ETO truncated
AF9	ALL1-fused gene from chromosome 9 protein
AMKL	Acute megakaryoblastic leukemia
AML	Acute myeloid leukemia
AML1	Acute myeloid leukemia 1
ANKs	Ankyrin repeats
B-ALL	B-cell acute lymphoblastic leukemia
CARM1	Coactivator-associated arginine methyltransferase 1

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CBF1	C promoter-binding factor 1
CBFβ	Core-binding factor β
CDK8	Cyclin-dependent kinase 8
CKII	Casein kinase II
CLL	Chronic lymphocytic leukemia
CoA	Coactivator
CoR	Corepressor
CSL	*Homo sapiens* CBF1, *Drosophila melanogaster* Suppressor of Hairless, and *Caenorhabditis elegans* Lag-1
CtBP	C-terminal-binding protein
CtIP	CtBP-interacting protein
DDX5	DEAD-box helicase 5
DLBCL	Diffuse large B-cell lymphoma
DLL1	DELTA-LIKE 1
DLL4	DELTA-LIKE 4
dnMAML1	dominant-negative MAML1
EBNA2	Epstein-Barr virus nuclear antigen 2
EGR2	*Early growth response 2*
Ep300	E1A-binding protein P300
Ep400	E1A-binding protein P400
ESCs	Embryonic stem cells
ETO	Eight-twenty-one
FBXW7	F-Box and WD repeat domain-containing 7
FHL1C	Four-and-a-half LIM domain protein 1C
FLT3	FMS-like tyrosine kinase 3
GCN5	General control of amino acid synthesis protein 5-like 2
GoF	Gain of function
GSI	γ-secretase inhibitor
H2A.Zac	H2A.Z acetylation
HAT	Histone acetyltransferase
HD	heterodimerization domain
HDACs	Histone deacetylases
Hes1	*Hairy and Enhancer of Split 1*
HPCs	Hematopoietic progenitor cells
KAT	lysine acetyltransferase
KAT2A	lysine acetyltransferase 2A
KAT2B	lysine acetyltransferase 2B
KAT3B	lysine acetyltransferase 3B
KAT5	lysine acetyltransferase 5
KBF2	H-2 K binding factor-2
KDM1A	lysine demethylase 1A
KDM5A	lysine demethylase 5A
KDM7B	lysine demethylase 7B
KMT2A	lysine-specific methyltransferase 2A
KMT2D	lysine-specific methyltransferase 2D
L3MBTL3	lethal (3) malignant brain tumor-like protein 3
LID	Little imaginal discs
LoF	Loss of function
LSD1	lysine-specific demethylase 1
Lz	Lozenge
MAL	Megakaryocytic acute leukemia
MAM	Mastermind
MAML	Mastermind-like
MCL	Mantle cell lymphomas
MINT	MSX2-interacting protein
MKL1	Megakaryoblastic leukemia 1
MLL	Mixed-lineage leukemia
MS	Mass spectrometry
MTG16	Myeloid translocation gene on chromosome 16 protein
MTG8	Myeloid translocation gene on 8q22
MTGR1	Myeloid translocation gene-related protein 1
NACK	Notch activation complex kinase
NCoR	Nuclear receptor corepressor
NFAT	*Nuclear factor of activated T-cells*
NF-κB1	Nuclear factor kappa B subunit 1
NHR	Nervy homology regions
NICD	NOTCH intracellular domain
NICD1	NOTCH1 intracellular domain 1
NK	Natural killer
OTT	One twenty-two
PCAF	Ep300-CBP-associated factors
PEST	Proline, glutamic acid, serine, and threonine
PHF8	PHD finger protein 8
PRMT4	Protein arginine methyltransferase 4
PTMs	Posttranslational modifications
RBM15	RNA-binding motif protein 15
RBPID	RBPJ-interacting domain
RBPJ	Recombination signal-binding protein for immunoglobulin kappa J region
RBS	RBPJ-binding sites
RHD	Runt homology domain

Runx	Runt-related transcription factor
RUNX1	Runt-related transcription factor 1
SHARP	SMRT and HDACs-associated repressor protein
SMRT	Silencing mediator for retinoid and thyroid receptor
SMZL	Splenic marginal zone lymphomas
Spen	split ends
SPOC	Spen paralog and ortholog C-terminal
SPOCome	SPOC interactome
SRA	*Steroid receptor coactivator*
SuH	Suppressor of Hairless
TAD	Transactivation domain
T-ALL	T-cell acute lymphoblastic leukemia
TFs	transcription factors
Tip60	HIV-1 Tat-interactive protein, 60 kDa
UTR	Untranslated region
WT	Wild type
ZnF	Zinc fingers

Introduction

Notch signaling is an evolutionary highly conserved pathway that plays a pivotal role in many cellular and developmental processes including T-cell development (Vijayaraghavan and Osborne 2018) and angiogenesis (Pitulescu et al. 2017; Tetzlaff and Fischer 2018). Although *Notch* was originally described as a neurogenic gene in *Drosophila melanogaster*, the first analysis of *Drosophila* embryos made it clear that Notch signals are pleiotropic, affecting many tissues. After the cloning and sequencing of the *Notch* gene in the 1980s, it became clear that Notch is a single-pass transmembrane receptor. Subsequently, the *NOTCH1* gene was described to be a hotspot for chromosomal translocations in human T-cell acute lymphoblastic leukemia (T-ALL) (Ellisen et al. 1991). By now, we know that *NOTCH1* mutations are found not only in human T-ALL (Weng et al. 2004) but also in other forms of human leukemia, for example, chronic lymphocytic leukemia (CLL) (Puente et al. 2011) as well as many other cancer types (Giaimo and Borggrefe 2018).

At the molecular level, Notch signal transduction bears some unique features not seen in other pathways like TGFβ, Wnt, or Hedgehog signaling [also reviewed in Borggrefe et al. 2016]. For example, the Notch pathway does not involve any second messengers. Notch signaling occurs through direct interactions between the Notch receptor and its ligand exposed on neighboring cells (Fig. 2.1). Upon ligand binding, the extracellular protease cleavage site of the receptor is exposed and cleaved by ADAM (a disintegrin and metalloproteinase) proteases. Subsequently, a second cleavage of the receptor is mediated by a γ-secretase-containing complex leading to the release of the Notch intracellular domain (NICD), which is itself a transcriptional coactivator (Fig. 2.1). The NICD migrates into the nucleus and functions as a transcriptional coactivator together with RBPJ and mastermind (MAM) [reviewed in Oswald and Kovall 2018]. The transcription factor RBPJ is a central molecular switch in the Notch pathway and mediates either transcriptional repression or activation of Notch target genes (Fig. 2.1).

Transcription Factor RBPJ in Balancing Notch Target Gene Expression

Historically, RBPJ was discovered thirty years ago and was originally named RBPJκ [recombination signal binding protein for immunoglobulin kappa J region, (Hamaguchi et al. 1989)]. It also has different names, such as CBF1 (C promoter binding factor 1) or KBF2 [H-2K binding factor-2, (Brou et al. 1994)] and belongs to the CSL (*Homo sapiens* CBF1, *Drosophila melanogaster* Suppressor of Hairless and *Caenorhabditis elegans* Lag-1) protein family. The DNA binding sequence was identified as 5′-CGTGGGAA-3′ (Tun et al. 1994) and recent studies investigated the genome-wide distribution of RBPJ in several different tissues (Dieguez-Hurtado et al. 2019; Petrovic et al. 2019; Wang et al. 2011; Xie et al.

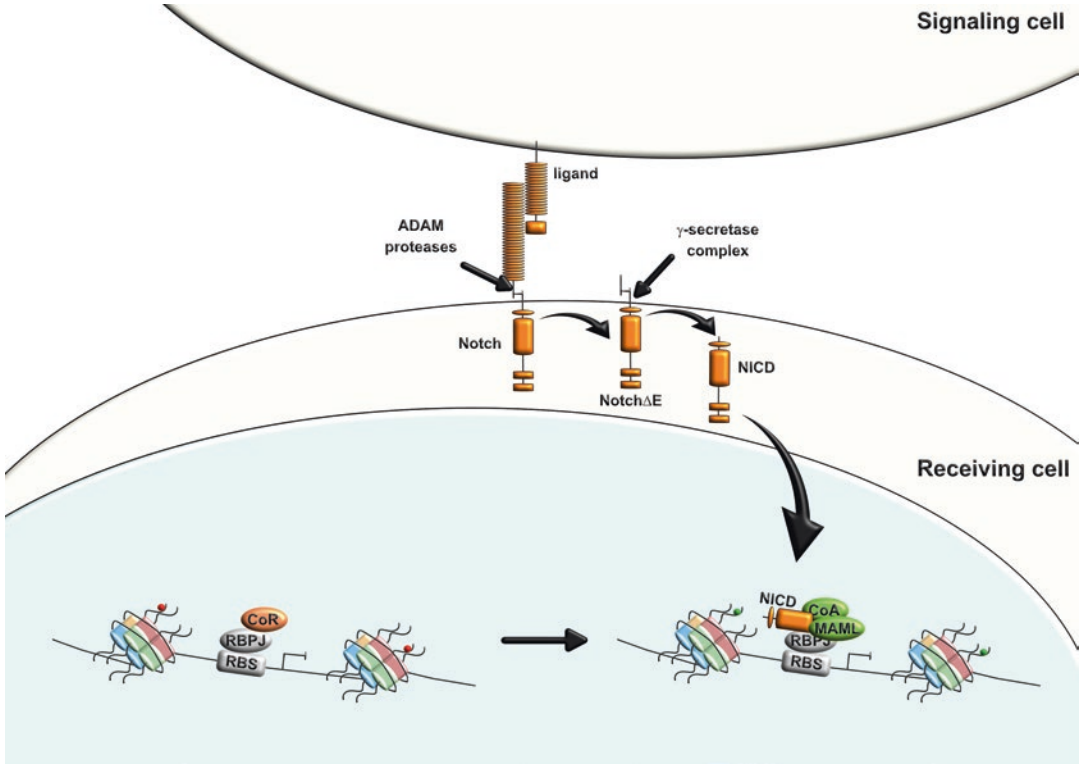

Fig. 2.1 The Notch signaling cascade. In absence of Notch signaling, the DNA-binding protein RBPJ is bound at the RBPJ-binding sites (RBS) where it recruits corepressors (CoR) preventing the expression of Notch target genes. The binding of ligands to Notch receptors induces a conformational change that allows their proteolytic cleavage by ADAM proteases producing an intermediate product known as NotchΔE. Subsequently, a γ-secretase-containing complex catalyzes a second cleavage of the Notch receptor releasing the Notch intracellular domain (NICD). The free NICD moves into the nucleus where it interacts with RBPJ and forms a trimeric complex together with Mastermind-like (MAML). This trimeric complex recruits additional coactivators (CoA) finally promoting expression of Notch target genes. Finally, proteasome-dependent degradation of the NICD terminates the signal, and the RBPJ-associated corepressor complex is reassembled at the RBS inducing repression of Notch target genes. Green and red balls indicate positive and negative histone marks, respectively

2016; Zhao et al. 2011). RBPJ shares some structural similarities with Rel Homology Domain proteins such as NF-κB1 (nuclear factor kappa B subunit 1) and NFAT [*nuclear factor of activated T-cells,* (Kovall and Hendrickson 2004)]. It is the centerpiece of transcriptional regulation in Notch signaling, acting as a molecular hub for interactions of either corepressor or coactivators. In the absence of a Notch signal, RBPJ interacts with the cofactor SHARP recruiting histone deacetylase-containing corepressor complexes. In the presence of a Notch signal, a ternary complex containing RBPJ, NICD and MAM-like (MAML) is assembled and expression of Notch target genes is induced (Fig. 2.1). The RBPJ/NICD/MAML-containing coactivator complex also recruits lysine acetyltransferases (KATs) such as KAT3B/Ep300 (lysine acetyltransferase 3B/E1A binding protein P300), KAT2B/PCAF (lysine acetyltransferase 2B/Ep300-CBP-associated factors) and KAT2A/GCN5 [lysine acetyltransferase 2A/ general control of amino acid synthesis protein 5-like 2, (Kurooka and Honjo 2000; Oswald et al. 2001)]. Interestingly, RBPJ was initially described as a repressor of transcription and its role as a molecular switch was further underscored by the finding that repression and activation via RBPJ involves the recruitment of distinct protein complexes [reviewed in (Borggrefe and Oswald 2009]. From

Table 2.1 List of well-defined interactors of the main components of the Notch signaling pathway: L3MBTL3, NICD, RBPJ, and SHARP

Interactor	Reference(s)	Structure
L3MBTL3 interactors		
KDM1A/LSD1	Xu et al. 2017	n.d.
NICD interactors		
CARM1/PRMT4	Hein et al. 2015	n.d.
Cyclin C/CDK8	Fryer et al. 2004	n.d.
DDX5	Jung et al. 2013; Lin et al. 2013	n.d.
Ep400/Tip60 complex	Giaimo et al. 2018	n.d.
KAT2A/GCN5	Kurooka and Honjo 2000	n.d.
KAT2B/PCAF	Kurooka and Honjo 2000	n.d.
KAT3B/Ep300	Oswald et al. 2001	n.d.
MAML	Wu et al. 2000	Nam et al. 2006; Wilson and Kovall 2006
NACK	Jin et al. 2017; Weaver et al. 2014	n.d.
SRA	Jung et al. 2013	n.d.
RBPJ interactors		
EBNA2	Grossman et al. 1994; Henkel et al. 1994; Ling et al. 1993; Waltzer et al. 1994; Zimber-Strobl et al. 1994	n.d.
Ep400/Tip60 complex	Giaimo et al. 2018	n.d.
Ikaros	Geimer Le Lay et al. 2014	n.d.
KDM5A/LID	Liefke et al. 2010	n.d.
KDM7B/PHF8	Yatim et al. 2012	n.d.
KyoT2/FHL1C	Taniguchi et al. 1998	Collins et al. 2014
KyoT3/FHL1B	Liang et al. 2008	n.d.
L3MBTL3	Xu et al. 2017	n.d.
NICD	Fortini and Artavanis-Tsakonas 1994	Nam et al. 2006; Wilson and Kovall 2006
RBM15/OTT	Ma et al. 2007	n.d.
SHARP	Oswald et al. 2002	Yuan et al. 2019
RITA	Wacker et al. 2011	Tabaja et al. 2017
RTA	Liang et al. 2002	n.d.
SHARP interactors		
AML1/ETO	Salat et al. 2008; Thiel et al. 2017	n.d.
CtIP/CtBP	Oswald et al. 2005	n.d.
KMT2D	Oswald et al. 2016	n.d.
MTG8/ETO	Salat et al. 2008	n.d.
MTG16	Engel et al. 2010	n.d.
MTGR1	Engel et al. 2010	n.d.
NCoR	Oswald et al. 2016	n.d.

these studies a model emerged (Fig. 2.1) stating that presence of NICD converts the RBPJ-corepressor to the RBPJ-NICD-coactivator complex (Borggrefe and Oswald 2009; Bray 2006).

In the recent years, the RBPJ interactome has been extensively studied [(Borggrefe and Liefke 2012; Guruharsha et al. 2014; Ho et al. 2018; Yatim et al. 2012) and Table 2.1] in order to understand at the molecular level how gene repression and activation are regulated. As part of the corepressor complex, RBPJ can directly interact with corepressor SHARP [SMRT (silencing

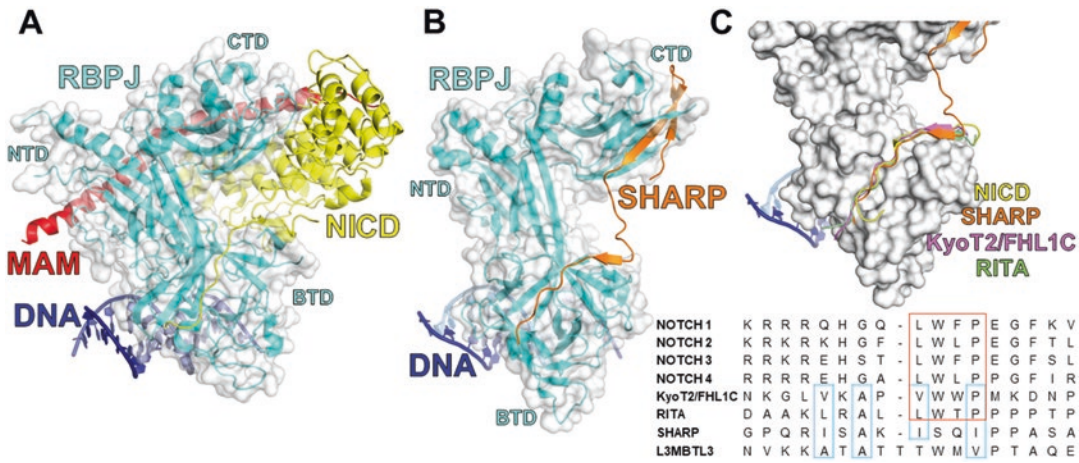

Fig. 2.2 Overview on the known crystal structures of RBPJ-associated complexes. (**a**) The structure of the *Caenorhabditis elegans* RBPJ/NICD/MAML ternary activation complex (PDBID: 2FO1). RBPJ, shown in cyan with a transparent white surface, consists of three major domains. The N-terminal domain (NTD) makes direct contacts primarily with MAML (red) and DNA (blue). The beta-trefoil domain (BTD) interacts with DNA and NICD (yellow). The C-terminal domain (CTD) interacts with MAML and NICD. (**b**) The structure of the *Mus musculus* RBPJ/SHARP repressor complex (PDBID: 6DKS). Like NICD, SHARP (orange) also binds the CTD and BTD of RBPJ. (**c**) Top: Structural representation of multiple corepressors that bind the BTD of RBPJ similarly. NICD (yellow) PDBID, 3 V79; SHARP (orange) PDBID, 6DKS; KyoT2/FHL1C (pink) PDBID, 4J2X; and RITA (light green) PDBID, 5EG6. Bottom: Multiple sequence alignments of coregulators that bind the BTD of RBPJ. Boxed in red is the highly conserved hydrophobic tetrapeptide seen in all four mammalian Notch isoforms as well as some corepressors. The blue boxes represent other highly conserved hydrophobic residues seen in multiple corepressors

mediator for retinoid and thyroid receptor) and HDACs (histone deacetylases)-associated repressor protein], also known as mouse MINT (MSX2-interacting protein) or Spen [split ends (Oswald et al. 2002)]. Recently, we determined the binding surfaces of the RBPJ/SHARP interaction at atomic resolution using X-ray crystallography [Fig. 2.2 (Yuan et al. 2019)]. Based on the RBPJ/SHARP structure, we could design a dominant-negative form of SHARP in a Notch-OFF state (Giaimo et al. 2017a; Xu et al. 2017; Yuan et al. 2019). When overexpressing the wild-type (WT) form of the RBPJ-interacting domain (RBPID) of SHARP, derepression of Notch target genes was observed; however, this was not the case when, based on the crystal structure, we mutated two amino acids within this domain (Yuan et al. 2019). Previous studies linked the repressive activity of SHARP to HDACs (Oswald et al. 2002; Oswald et al. 2016), and, in line with these studies, we observed increased histone acetylation upon overexpression of the WT but not the mutant RBPID (Yuan et al. 2019). Importantly, RBPJ depletion leads to derepression of Notch target genes in the same setting. This phenotype is efficiently rescued by a WT RBPJ but not a mutant in which the residues required for its interaction with SHARP are mutated (Yuan et al. 2019).

SHARP is a protein of more than 400 kDa characterized by a highly conserved SPOC (Spen paralog and ortholog C-terminal) domain which has a strong transcriptional repressive activity that depends on CtIP/CtBP (CtBP-interacting protein/C-terminal-binding protein) (Oswald et al. 2005). To better dissect the mechanism of the RBPJ/SHARP-mediated transcriptional repression, we have recently characterized, by mass spectrometry (MS), the SPOC interactome (SPOCome) (Oswald et al. 2016). This approach identified the HDACs-containing NCoR (nuclear receptor corepressor) complex, explaining how HDACs are recruited to RBPJ-bound enhancer sites; however, it also identified the KMT2D (lysine-specific methyltransferase 2D) complex

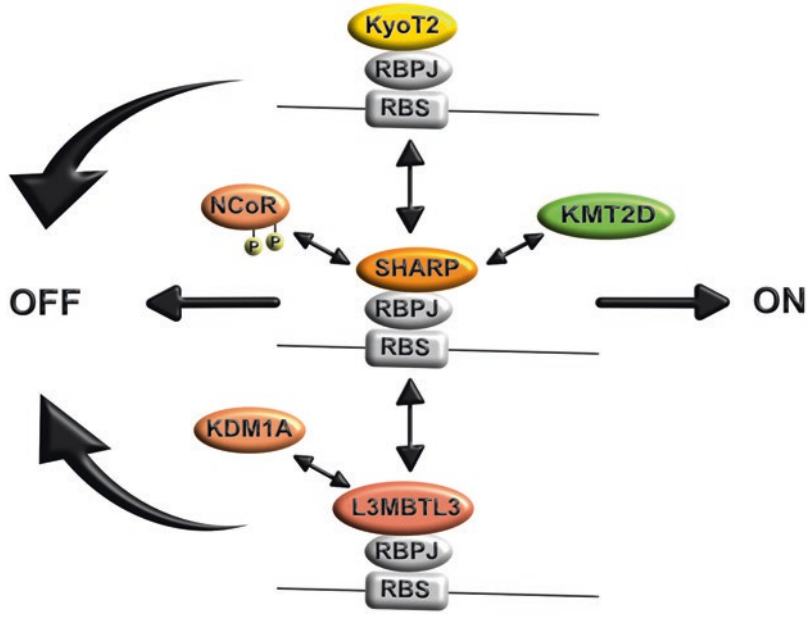

Fig. 2.3 Intermediate states involved in the transcriptional regulation of Notch target genes. The different RBPJ-associated corepressor complexes (SHARP, L3MBLT3, and KyoT2) are recruited at the same RBPJ-binding sites (RBS) in a well-defined temporal order and/or tissue-specific manner to promote repression of Notch target genes. Of note, SHARP can interact with the HDAC-containing NCoR corepressor complex when it is phosphorylated on two serine residues. NCoR recruitment moves the balance toward repression alternatively; NCoR interacts with the KMT2D-containing complex moving the balance toward gene activation. L3MBTL3 bridges the histone demethylase KDM1A/LSD1 (indicated as KDM1A) to RBPJ at RBS

(Oswald et al. 2016). This finding was quite unexpected as the KMT2D complex is involved in transcriptional activation in contrast to the repressive activity of the SPOC domain of SHARP. Previous structural studies of SMRT, the ortholog of NCoR, in complex with the SPOC domain of SHARP unveiled that this interaction depends on the phosphorylation of highly conserved serine residues of SMRT by casein kinase II (CKII) (Mikami et al. 2013; Mikami et al. 2014). Of note, NCoR is also phosphorylated on serine residues at its C-terminus (Yoo et al. 2012, 2013), and we found that NCoR phosphorylation is required for its interaction with the SPOC domain and dependent on CKII [(Oswald et al. 2016) and Fig. 2.3]. KMT2D and NCoR are in competition for binding to SPOC, and phospho-NCoR displaces KMT2D leading to transcriptional repression [(Oswald et al. 2016) and Fig. 2.3]. These data support the hypothesis that SHARP, integrating different stimuli, acts as a poising factor for Notch target genes, balancing repressive and activating histone marks [(Giaimo et al. 2017b; Oswald et al. 2016) and Fig. 2.3].

The SPOC domain of SHARP directly interacts with ETO [eight-twenty-one, also known as MTG8 (myeloid translocation gene on 8q22)] which acts as a corepressor of Notch target genes by meaning of deacetylation (Salat et al. 2008). ETO is a member of the MTG family of corepressors of transcription which includes also MTGR1 (myeloid translocation gene-related protein 1) and MTG16 (myeloid translocation gene on chromosome 16 protein). Both MTGR1 and MTG16 interact with RBPJ; however, only MTG16 is displaced from RBPJ by the NOTCH1 intracellular domain 1 (NICD1) (Engel et al. 2010). In conclusion, the SPOC domain of SHARP is able to interact with several different proteins. It remains to be investigated how the

varying interaction partners are recruited to enhancers. Our favorite working hypothesis is that posttranslational modifications (PTMs) such as phosphorylation determine specificity in terms of composition and strengths of recruitment of corepressors. In that regard, it is appealing that the highly conserved SPOC domain interacts with double-phosphorylated peptide (Oswald et al. 2016). This might be also the case for other SPOC-interaction partners. Interestingly, RBM15 [RNA-binding motif protein 15, also known as OTT (one twenty-two)], another SPOC domain-containing protein, was shown to modulate the Notch signaling pathway in a cell-type-specific fashion (Ma et al. 2007), marking the importance of the SPOC domain-containing proteins in the regulation of the Notch signaling pathway.

Another direct interactor of RBPJ is KyoT2 [also known as FHL1C (four-and-a-half LIM domain protein 1C) in human] which, competing with the NICD1, represses transcription of target genes (Collins et al. 2014; Taniguchi et al. 1998). The structure of RBPJ in complex with KyoT2/FHL1C reveals a good overlap with the RBPJ/SHARP and RBPJ/Notch binding surfaces [Fig. 2.2 (Collins et al. 2014; Yuan et al. 2019)]. One more isoform, KyoT3/FHL1B, is also able to interact with RBPJ and to promote gene repression (Liang et al. 2008), but this is not the case for the isoform KyoT1/FHL1A (Taniguchi et al. 1998). It remains to elucidate whether the repressive mechanism of KyoT2/FHL1C and KyoT3/FHL1B is exclusively based on their competition with the NICD or whether it depends on other cofactors, for example, a link between KyoT2/FHL1C and Polycomb has been proposed (Quin et al., PMID, 14999091, and Quin et al., 15,710,417).

As part of the corepressor complex, RBPJ recruits histone demethylase activities such as KDM5A/LID [lysine demethylase 5A/little imaginal discs (Di Stefano et al. 2011; Liefke et al. 2010; Moshkin et al. 2009)] and KDM1A/LSD1 [lysine demethylase 1A/lysine-specific demethylase 1 (Di Stefano et al. 2011; Mulligan et al. 2011; Xu et al. 2017; Yatim et al. 2012)]. KDM5A/LID directly interacts with RBPJ and demethylates H3K4me3 at RBPJ-bound enhancer sites promoting repression of Notch target genes (Liefke et al. 2010), while KDM1A/LSD1 indirectly interacts with RBPJ via L3MBTL3 [lethal (3) malignant brain tumor-like protein 3] (Xu et al. 2017). We identified L3MBTL3 in a screen for RBPJ interactors and observed that the L3MBTL3-RBPJ interaction is conserved in *Drosophila melanogaster* and *Caenorhabditis elegans* (Xu et al. 2017). Notably, L3MBTL3 and NICD1 bind to the same binding surface on RBPJ: While NICD1 displaces L3MBTL3 from RBPJ, the latter does not outcompete NICD1 for binding to RBPJ (Xu et al. 2017). The recruitment of KDM1A/LSD1 via L3MBTL3 is required to modulate H3K4 methylation states at RBPJ-bound enhancers promoting repression of target genes (Xu et al. 2017). In line with that, pharmacological inhibition of KDM1A/LSD1 leads to upregulation of Notch target genes (Augert et al. 2019); however it must be marked that, at least in lung cancer cells, KDM1A/LSD1 may indirectly regulate the Notch signaling pathway via a direct regulation of the expression of *NOTCH1* (Augert et al. 2019). A previous study linked KDM1A/LSD1 to repression of Notch target genes, but the authors also observed that KDM1A/LSD1 associates with the NOTCH1 coactivator complex to modulate H3K9 methylation states and finally promoting expression of Notch target genes (Yatim et al. 2012). Altogether, these data suggest that KDM1A/LSD1 acts as both an activator and a repressor of the Notch-dependent gene expression program. Finally, the demethylase KDM7B/PHF8 (lysine demethylase 7B/PHD finger protein 8) is also part of the NOTCH1 coactivator complex and supports expression of Notch targets by modulating H3K27 methylation states (Yatim et al. 2012).

Based on the available structural and biophysical data, SHARP, KyoT2/FHL1C, and L3MBTL3 interact in a mutually exclusive fashion with RBPJ: Different intermediate complexes may be dynamically recruited at the same enhancer in a defined temporal order or in a tissue-specific manner to modulate the chromatin structure leading to gene repression (Fig. 2.3). Since the RBPJ-associated cofactors interactions are strong and the DNA-binding affinity of RBPJ

is relatively weak, it can be assumed that the different RBPJ complexes are constantly exchanging, explaining how the different cofactors are recruited at a defined enhancer.

The activation of the Notch pathway leads to the release of the NICD from the cell membrane which, upon nuclear translocation, converts RBPJ from a repressor to an activator of transcription via the recruitment of additional coactivators (Fig. 2.1). One of the most important members of the coactivator complex is MAM which, together with RBPJ and NICD, forms a trimeric complex indispensable for activation of Notch target genes (Friedmann et al. 2008; Fryer et al. 2002; Kitagawa et al. 2001; Nam et al. 2006; Wilson and Kovall 2006; Wu et al. 2000, 2002). The human Mastermind family (Mastermind-like or MAML) consists of three members, all of them able to support Notch-dependent transcription (Lin et al. 2002). Probably, the most important function of MAML is to recruit the histone acetyltransferase (HAT) KAT3B/Ep300 to Notch target genes that supports gene expression via histone acetylation (Fryer et al. 2002; Jung et al. 2013; Oswald et al. 2001; Tottone et al. 2019; Wallberg et al. 2002). Additionally, KAT3B/Ep300 acetylates MAML leading to the recruitment of the coactivator NACK (Notch activation complex kinase) at Notch target genes (Jin et al. 2017; Weaver et al. 2014). MAML also recruits the cyclin C/CDK8 (Cyclin-dependent kinase 8) complex that phosphorylates the NICD leading to its proteasome-dependent degradation (Fryer et al. 2004). Another component of the Notch coactivator complex is the RNA helicase DDX5 (DEAD-box helicase 5) which, interacting with the long noncoding RNA *SRA* (*steroid receptor coactivator*), supports the recruitment of KAT3B/Ep300 and subsequent activation of Notch target genes (Jung et al. 2013; Lin et al. 2013). Furthermore, CARM1/PRMT4 (coactivator-associated arginine methyltransferase 1/protein arginine methyltransferase 4) promotes the activation of Notch target genes by arginine methylation of the NICD1 itself (Hein et al. 2015).

Recently, we characterized the interactome of the cleaved, active NICD1 in mouse progenitor cells (Giaimo et al. 2018). Using this approach, we identified the Ep400-KAT5/Tip60 (E1A-binding protein P400-lysine acetyltransferase 5/HIV-1 Tat-interactive protein, 60 kDa) complex (hereafter referred to as Ep400/Tip60 complex) as an NICD1 interactor. This complex attracted our attention as its subunits Ep400 and KAT5/Tip60 have been previously linked to deposition (Gevry et al. 2007) and acetylation (Kusch et al. 2004) of the histone variant H2A.Z, respectively. H2A.Z has been linked to several processes including heterochromatin regulation, DNA repair, and gene transcription both in a positive and negative fashion (Giaimo et al. 2019). We found that H2A.Z depletion leads to upregulation of Notch target genes, and this enhanced expression is associated with increased active marks, namely, H3K4me2 and H3K27ac, at Notch-dependent enhancer elements. These data suggest H2A.Z as a negative regulator of Notch target genes, and this conclusion is further supported by the observation that activation of Notch signaling leads to decreased H2A.Z occupancy at Notch-dependent enhancers (Giaimo et al. 2018). However, while H2A.Z occupancy negatively correlates with induction of Notch target genes, acetylation of H2A.Z (H2A.Zac) does it in a positive manner suggesting that H2A.Z is involved in both gene repression and activation and the difference between the two functions is obtained via its acetylation. Overexpression of H2A.Z leads to upregulation of Notch target gene *Hairy and Enhancer of Split 1* (*Hes1*), while this upregulation is more modest when an acetylation-defective H2A.Z mutant is overexpressed (Giaimo et al. 2018). Our data further indicate that acetylation of H2A.Z is highly dynamic, which also reconciles previous contrasting results showing H2A.Z as a repressor or an activator of transcription (Gevry et al. 2007, 2009; Giaimo et al. 2019). We observed that Ep400 interacts with RBPJ and it is recruited to Notch-dependent enhancers in a Notch-dependent fashion (Giaimo et al. 2018). Furthermore, making use of a tethering approach, we could show that Tip60 promotes H2A.Zac supporting gene expression

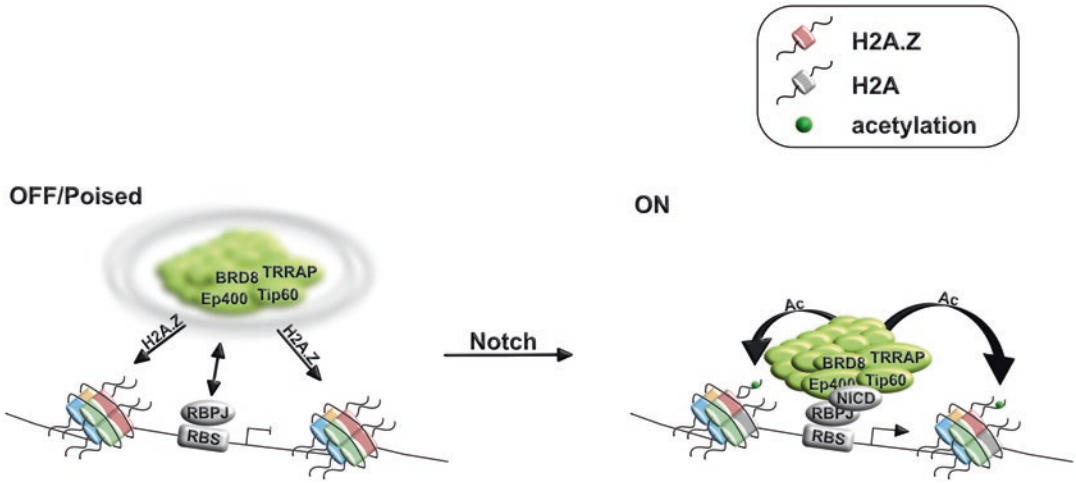

Fig. 2.4 Model for the regulation of Notch target gene by the Ep400/Tip60 complex and histone variant H2A.Z. In the repressed (OFF) or poised state, RBPJ directly interacts with Ep400 leading to the recruitment of the Ep400/Tip60 complex. This interaction is unstable but sufficient to promote deposition of the histone variant H2A.Z. Upon activation of the Notch pathway (ON), the Notch intracellular domain (NICD) directly interacts with RBPJ and Ep400 leading to stabilization of the RBPJ-Ep400 interaction. In turn, this results in acetylation of H2A.Z (indicated with green balls) and finally gene activation

(Giaimo et al. 2018). In summary, our data suggest that in a Notch-OFF or poised state, the Ep400/Tip60 complex is recruited to Notch-dependent enhancer sites via an unstable interaction with RBPJ promoting loading of H2A.Z. Upon Notch activation, the interaction between the Ep400/Tip60 complex and RBPJ is stabilized via additional interactions with the NICD1 protein promoting acetylation of H2A.Z and finally gene expression (Fig. 2.4).

The classical model for the regulation of Notch target genes suggests that RBPJ is persistently bound to its cognate sequences promoting gene repression or activation based on the stimulation of the NOTCH receptor. However, recent studies challenged this model and suggest that RBPJ is weakly bound to its enhancers in absence of stimulus, while its genomic occupancy significantly increases upon activation of the Notch pathway and landing of the NICD at target enhancers (Fig. 2.5). Earlier studies in *Drosophila melanogaster* cell lines observed increased occupancy of Su(H) (Suppressor of Hairless), the *Drosophila* homolog of RBPJ, upon induction of Notch signaling (Krejci and Bray 2007). Recently, single-molecule tracking in vivo studies allowed to define that Su(H) transiently binds the DNA in the OFF state (Gomez-Lamarca et al. 2018). Upon Notch activation, the DNA binding of Su(H) significantly increases (Gomez-Lamarca et al. 2018).

Similarly to *Drosophila*, activation of the Notch pathway leads to increased RBPJ occupancy in mammalian cell lines (Castel et al. 2013; Wang et al. 2014; Yashiro-Ohtani et al. 2014); however, Castel and colleagues identified two different classes of RBPJ-binding sites: dynamic sites at which RBPJ is bound only upon Notch activation and static sites at which RBPJ is bound independently of the Notch activation (Castel et al. 2013). To note, NICD binding occurs exclusively at the dynamic but not static sites, and furthermore, Castel and colleagues observed that RBPJ depletion leads to derepression of few genes associated with static sites and about 50% of the genes associated with dynamic sites (Castel et al. 2013). However, this analysis uses different cell lines for ChIP-Seq (C2C12 cell lines) and gene expression analysis (quiescent satellite cells) (Castel et al. 2013). As a consequence, we do not know whether all the derepressed genes assumed to be associated with static or dynamic RBPJ sites are so. The DNA-binding strength of RBPJ does not seem to be regulated exclusively

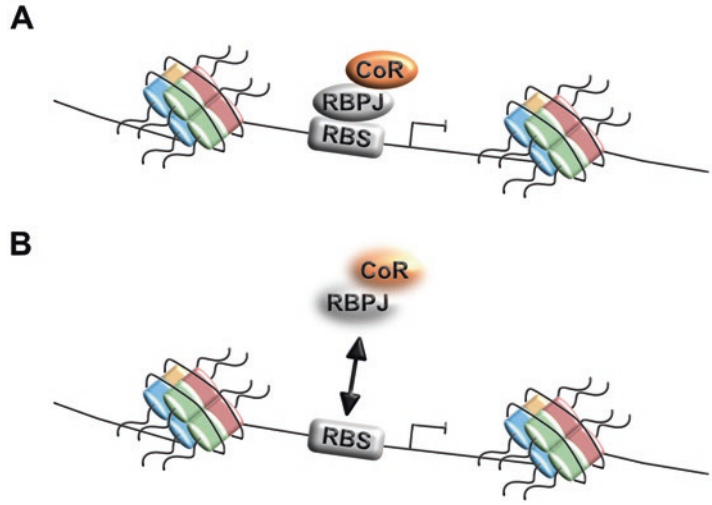

Fig. 2.5 New model for regulation of Notch target genes. (**a**) The classic model for regulation of Notch target genes is based on the binding of RBPJ at its cognate RBPJ-binding sites (RBS) in absence of Notch signaling. In this scenario, RBPJ recruits corepressors (CoR) preventing expression of target genes. (**b**) New data suggest that RBPJ transiently binds to the RBS in absence of Notch signaling promoting gene repression. Upon Notch activation, the cleaved Notch intracellular domain (NICD) interacts with RBPJ leading to increased DNA binding of RBPJ at the RBS. This event leads finally to activation of Notch target genes

by the NICD, for example, a recent study proposed that HDAC1 and KDM5A/LID play a negative and positive role, respectively, in this process, at least in mitosis (Dreval et al. 2019). Similarly, the BRM complex promotes Su(H) binding in *Drosophila* (Pillidge and Bray 2019). The above findings implicate dynamic binding of RBPJ depending on dynamic coactivator binding.

Promoter specificity might also be achieved by the usage of mono- versus dimeric RBPJ-bound enhancers. In fact, several RBPJ enhancers are characterized by two correctly spaced and oriented binding motifs at which a dimeric NICD1/RBPJ/MAML complex is recruited (Hass et al. 2015; Nam et al. 2007; Severson et al. 2017). The structure of the dimeric NICD1/RBPJ/MAML complex has been solved (Arnett et al. 2010); however, we do not know the impact of PTMs of the NICD on the dimeric NICD1/RBPJ/MAML complex formation (Borggrefe et al. 2016).

The DNA binding of RBPJ is also dependent on other transcription factors (TFs). This is, for example, marked by the loss of function (LoF) of Lozenge (Lz), the *Drosophila* homolog of Runx (Runt-related transcription) factors, which results in reduced SuH occupancy (Terriente-Felix et al. 2013). Similarly, Lz overexpression leads to increased SuH occupancy and increased response to Notch activation (Skalska et al. 2015). In line with that, the Runx DNA-binding motif is found near the RBPJ-binding sites (Wang et al. 2011), and RBPJ and RUNX1 [(Runt-related transcription factor 1) also known as AML1 (acute myeloid leukemia 1)] colocalize genome-wide (Wang et al. 2014). In *Drosophila*, the basic helix-loop-helix (bHLH) TFs Twist and Dorsal prime RBPJ-dependent enhancers leading to synchronized and sustained enhancer activity (Falo-Sanjuan et al. 2019), marking the importance of tissue-specific TFs in the Notch response. Additionally, RBPJ colocalizes and interacts with the DNA-binding protein IKAROS which is required for repression of Notch target genes (Geimer Le Lay et al. 2014). However, the exact relationship between RBPJ and IKAROS is not clear, and we do not know whether their interaction is required to support the DNA binding of RBPJ and vice versa. This can be addressed by performing depletion of IKAROS followed by ChIP versus RBPJ and the other way round.

Notch Signaling in Hematopoiesis

The essential role for Notch signaling in inducing T-cell development has been extensively investigated [reviewed in Vijayaraghavan and Osborne 2018]. Inducible depletion of the *Notch1* gene in bone marrow precursors results in a block of T-cell development associated with ectopic appearance of donor progenitor-derived $B220^+$ immature B-cells in the thymus (Radtke et al. 1999; Wilson et al. 2001). Accordingly, RBPJ conditional knockout mice are characterized by a block in T-cell development associated with appearance of B-cells in the thymus (Han et al. 2002). Similar results were obtained by gain of function (GoF) of the Notch target gene *Deltex1* (Izon et al. 2002) or by overexpression of a dominant-negative MAML1 (dnMAML1) (Maillard et al. 2004). These data suggest Notch signaling as the driver of the T-cell differentiation program, and in line with that, retroviral expression of the NICD in murine hematopoietic precursors, followed by transplantation into recipient mice, leads to an abnormal appearance of immature double-positive T-cells in the bone marrow and subsequent development of T-cell leukemia, while B-cell development is blocked (Pear et al. 1996; Pui et al. 1999). Furthermore, MTG16 knockout results in defects in T-cell differentiation both in mice and using $MTG16^{-/-}$ hematopoietic progenitors (Engel et al. 2010; Hunt et al. 2011).

The critical role of Notch signaling in activating the T-cell lineage differentiation program is also marked by the observation that the engagement of NOTCH receptors by DELTA-LIKE 1 (DLL1) ligand expressed on the surface of OP9 stromal cells leads to the differentiation of hematopoietic progenitor cells (HPCs) and embryonic stem cells (ESCs) into T-cells (Schmitt et al. 2004b; Schmitt and Zuniga-Pflucker 2002). The thymus represents a nonpermissive environment for the development of myeloid, natural killer (NK), and B-cells because the thymic epithelium offers the DLL1 and DLL4 ligands to the NOTCH1-expressing progenitor T-cells (Feyerabend et al. 2009; Schmitt et al. 2004a). Of note, Notch blocks the alternative differentiation pathways even if the cells are ectopically forced to express TFs required for differentiating versus other lineages (Franco et al. 2006; Laiosa et al. 2006).

Notch Signaling in Leukemia

Given the key role of Notch signaling in T-cell differentiation, it is not surprising to observe aberrant regulation of the Notch pathways in T-cell leukemias. Mutations of the *NOTCH1* gene have been identified in T-ALL patients and cell lines (Breit et al. 2006; Larson Gedman et al. 2009; Mansour et al. 2006; Palomero et al. 2006; Weng et al. 2004). These mutations lead to increased activation of the pathway, and they can be classified into two different groups based on the molecular mechanism: mutations that lead to ligand-independent activation of the pathway and mutations that increase the half-life of the NICD proteins. The former include translocations that fuse the NICD-encoding sequences to another gene or mutations that influence the cleavage of the receptor, for example, mutations of the heterodimerization domain (HD) (Larson Gedman et al. 2009; Malecki et al. 2006; Weng et al. 2004). The latter are mutations that result in C-terminal truncated NICD proteins lacking the PEST (proline, glutamic acid, serine, and threonine) domain which is required for the turnover of the NICD proteins (Larson Gedman et al. 2009; Palomero et al. 2006; Weng et al. 2004). Mutations, although very rare, may also occur in the ankyrin repeats (ANKs) and in the transactivation domain (TAD) of NOTCH1 in T-ALL (Zhu et al. 2006).

Importantly, hyperactivation of the Notch pathway can also be achieved compromising the activity of its negative regulators. In fact, mutations of the NOTCH1 E3-ubiquitin-ligase F-Box and WD repeat domain-containing 7 (FBXW7)-encoding gene have been identified in T-ALL (Larson Gedman et al. 2009). Additionally, upregulation of positive regulators of the Notch pathway can also lead to hyperactivation of the pathway. In line with this, *MAML2* is upregulated in B-cell-derived lymphomas (Kochert et al.

2011) and was described fused to the *lysine-specific methyltransferase 2A* (*KMT2A*) gene in T-ALL as result of a chromosomal inversion (Metzler et al. 2008). However, the Notch pathway, via HES1, seems to have a tumor-suppressive function in B-cell acute lymphoblastic leukemia [B-ALL (Kannan et al. 2011)].

Recently, activating *NOTCH* mutations of the PEST domain have been identified also in CLL (Fabbri et al. 2011; Puente et al. 2011). Interestingly, mutations of the 3´-UTR (untranslated region) of the *NOTCH1* gene have also been identified in CLL leading to increased activation of the pathway (Puente et al. 2015). Activating *NOTCH1* mutations significantly correlates with Richter transformation and chemorefractory CLL, and they have been proposed as predictors of poor survival (Fabbri et al. 2011). In line with a role for Notch signaling in CLL, γ-secretase inhibitor (GSI) treatment of B-CLL cells reduces their survival by meaning of apoptosis (Rosati et al. 2009). Similar conclusions were reached using antibodies directed against NOTCH receptors, and furthermore the same study observed that Notch signaling is involved in drug resistance (Nwabo Kamdje et al. 2012). Of note, *EGR2* (*early growth response 2*) mutations are frequently associated with *NOTCH1* or *FBXW7* mutations in CLL patients (Young et al. 2017). The observation that active Notch signaling is detectable also in CLL cases that lack NOTCH1 mutations suggests that other mechanisms can be used to activate the pathway in this disease and imply Notch signaling as a more general deregulated pathway associated with CLL (Fabbri et al. 2017).

In mantle cell lymphomas (MCL), activating mutations of the *NOTCH1* gene map to the HD- and PEST-encoding regions (Kridel et al. 2012), and recently a genome-wide study identified Notch targets and RBPJ-binding sites in MCL cell lines (Ryan et al. 2017). Similarly, truncating *NOTCH2* mutations were detected in MCL and diffuse large B-cell lymphoma (DLBCL) with the latter also characterized by missense *NOTCH2* mutations (Bea et al. 2013) and *NOTCH1* mutations (Fabbri et al. 2011). Activating *NOTCH2* and *NOTCH1* mutations were also described in splenic marginal zone lymphomas (SMZL) as well as inactivating *SHARP* mutations and mutations of other components of the Notch pathway (Rossi et al. 2012).

Mutations of the Notch pathway similar to the leukemia-associated ones have also been identified in solid tumors (Giaimo and Borggrefe 2018) marking the importance to develop new therapies aimed to target the Notch pathway.

Aberrant regulation of the Notch signaling pathway was also linked to the AML characterized by the t(8;21)(q22/q22) translocation that fuses the *AML1* (also known as *RUNX1*) gene to the *ETO* gene. *AML1* encodes for a hematopoietic cell-specific TF which heterodimerizes with a non-DNA-binding protein called CBFβ [core-binding factor β (Ogawa et al. 1993a, b)], and it is essential for definitive hematopoietic development (Okada et al. 1998; Okuda et al. 1996; Wang et al. 1996). AML1 is characterized by an N-terminal Runt homology domain (RHD) which characterizes all members of the RUNX family (RUNX1, RUNX2, and RUNX3) and by a C-terminal TAD. Both DNA binding and heterodimerization with CBFβ are mediated through the RHD, and the function of CBFβ is to increase the stability and the DNA-binding affinity of AML1 (Huang et al. 2001; Tahirov et al. 2001). On the other side, the *ETO* gene, highly expressed in the brain (Miyoshi et al. 1993) and in hematopoietic cells (Erickson et al. 1996), is the homolog of the *Drosophila* Nervy in four regions protein (Feinstein et al. 1995); in fact, it encodes for a non-DNA-binding protein characterized by four evolutionarily conserved functional domains called nervy homology regions (NHR): the NHR2 forms an amphipathic helix and it is important for homodimerization (Lutterbach et al. 1998a) and heterodimerization with MTGR1 (Kitabayashi et al. 1998); the NHR4 contains two putative zinc fingers (ZnF) required for interactions with NCoR and SMRT which links ETO to HDACs (Gelmetti et al. 1998; Lutterbach et al. 1998b; Wang et al. 1998). Of note, the function of NHR4 is strongly dependent on NHR2 (Zhang et al. 2001).

The t(8;21)(q22/q22) translocation fuses the DNA encoding the first N-terminal 177 residues

of AML1, which include the RHD, in frame with nearly all of ETO (Erickson et al. 1992; Kozu et al. 1993; Miyoshi et al. 1993; Nisson et al. 1992). This translocation leads to deletion of the C-terminal activation domain of AML1, and the resulting AML1/ETO (AE) protein acts as a dominant-negative form of AML1, which binds to AML1-binding sites (Gardini et al. 2008) repressing target genes (Frank et al. 1995; Liu et al. 2007). Of note, A/E expression requires additional mutations to induce leukemia in a murine in vivo model (Yuan et al. 2001), but this is not true for two different C-terminal truncated AE proteins [AML1/ETO 9a (AE9a) and AML1/ETO truncated (AEtr)] which are potent inducers of leukemia in mice (Yan et al. 2009; Yan et al. 2004; Yan et al. 2006). Interestingly, AE9a is deleted of NHR3 and NHR4, arguing against the well-accepted model that AE acts exclusively as a repressor of AML1 target genes (Heibert et al. 2001).

The first evidence about an aberrant regulation of the Notch signaling pathway in AML came out with the observation that overexpression of AE leads to upregulation of the Notch target gene *Hes1* (Alcalay et al. 2003). The underlying mechanism was unveiled when ETO was identified as a component of the RBPJ/SHARP corepressor complex (Salat et al. 2008). In detail, ETO and, surprisingly, AE directly interact with SHARP, but while ETO is able to augment SHARP-mediated repression, this is not the case for AE. Furthermore, knockdown of ETO or overexpression of AE resulted in activation of Notch target genes, suggesting that AE is able to derepress their expression, probably contributing to the oncogenic potential of AE in AML (Salat et al. 2008). In line with that, MTG16 which was also found fused to AML1 in cases of secondary AML cases (Gamou et al. 1998) interacts with the RBPJ-associated corepressor complex, and this interaction is regulated in a Notch-dependent fashion (Engel et al. 2010).

The exact role of CBFβ in the transformation process driven by AE remained unclear for a long time as two different studies arrived to opposite conclusions (Kwok et al. 2009, 2010; Park et al. 2009; Roudaia et al. 2009). Kwok and colleagues observed that the AE/CBFβ interaction is dispensable for leukemic transformation when CBFβ-interacting deficient AE mutants were retrovirally transduced into primary bone marrow cells (Kwok et al. 2009, 2010). In contrast, the study from Roudaia and colleagues observed that this interaction is strongly required to induce leukemia (Park et al. 2009; Roudaia et al. 2009) and in support of that, inhibitors of the AE/CBFβ interaction reduce cell proliferation of the ME-1 cell line characterized by a chromosomal translocation that involves the CBFβ-encoding gene (Gorczynski et al. 2007).

Our recent study helped to clarify these contradicting results. We designed CBFβ-interacting defective AE9a mutants, and upon retroviral transduction into HoxB4-immortalized hematopoietic progenitors, we observed that the AE/CBFβ interaction is required to derepress Notch target genes but not to deregulate AML1 target genes (Thiel et al. 2017). Furthermore, the AE/CBFβ interaction is required for the colony-forming potential of transduced progenitors and to induce leukemia into recipient mice, and it must be noted that mice receiving the CBFβ-interacting defective AE9a mutant present only with myeloproliferative defects (Thiel et al. 2017). These data suggest that AE9a deregulate AML1 targets independently of CBFβ leading to a myeloproliferative disease; however, the AE9a/CBFβ interaction is required to deregulate Notch target genes and induce leukemia.

While these data suggest that derepression of Notch signaling has an oncogenic role in AML, other studies observed the opposite in fact: Notch signaling has a tumor-suppressive role in AML cases that are not associated with the t(8;21) translocation (Kannan et al. 2013; Lobry et al. 2013). In this case, the tumor-suppressive role of Notch signaling seems to be dependent on the repressive activity of HES1 (Kannan et al. 2013; Tian et al. 2015b). In line with that, *HES1* expression correlates with a better prognosis in AML cases characterized by CBFβ alterations (Tian et al. 2015a), and pharmacological activation of Notch signaling has a tumor-suppressive function (Ye et al. 2016). In mouse models of MLL/AF9 (mixed-lineage leukemia/ALL1-fused gene from

chromosome 9 protein)-induced AML, HES1 has a tumor-suppressive role by promoting repression of FLT3 [FMS-like tyrosine kinase 3 (Kato et al. 2015; Lobry et al. 2013)]. Interestingly, Lobry and colleagues observed that Notch activation, in an AE background, has a tumor-suppressive role (Lobry et al. 2013). The discrepancy observed between our study (Thiel et al. 2017) and the study from Lobry and colleagues (Lobry et al. 2013) may be due to the different approaches used: while we only overexpressed AE9a leading to derepression, Lobry and colleagues overexpressed both AE and NICD2. In this way, the activation levels may bring the difference(s) between oncogenic and tumor-suppressive role for Notch signaling with a weak activation (derepression) having an oncogenic role and a stronger activation (given by the AE and the NICD2 together) having a tumor-suppressive role.

Of note, also the fusion protein OTT/MAL [one twenty-two/megakaryocytic acute leukemia, also known as RBM15/MKL1 (RNA-binding motif protein 15/megakaryoblastic leukemia 1)], which is the result of the t(1;22)(p13;q13) translocation, was proposed to disturb the repressive function of RBPJ in acute megakaryoblastic leukemia [AMKL (Mercher et al. 2009)].

Conclusion and Outlook

The function of the transcription factor RBPJ not only in the presence but also in the absence of Notch activation is of major importance, since this affects chromatin regulation and hence target specificity. It would be highly desirable to have novel compounds that specifically disrupt the RBPJ-corepressor function in certain disease settings like AML. Similarly, compounds able to disrupt the activation function of RBPJ would be very helpful to avoid the serious off-target effects observed with γ-secretase inhibitors. In line with that, a recent study characterized a new RBPJ inhibitor that prevents both its repressive and activating function (Hurtado et al. 2019). Chromatin regulation is to be expected at the center of RBPJ-mediated repressive mechanisms.

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Consent for Publication All authors have approved the manuscript.

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3

Unravelling of Hidden Secrets: The Tumour Suppressor Lethal (2) Giant Discs (Lgd)/CC2D1, Notch Signalling and Cancer

Tobias Reiff, Miriam Baeumers, Christine Tibbe, and Thomas Klein

Abstract

The endosomal pathway plays a pivotal role upon signal transduction in the Notch pathway. Recent work on *lethal (2) giant discs* (*lgd*) points to an additional critical role in avoiding uncontrolled ligand-independent signalling during trafficking of the Notch receptor through the endosomal pathway to the lysosome for degradation. In this chapter, we will outline the journey of Notch through the endosomal system and present an overview of the current knowledge about Lgd and its mammalian orthologs Lgd1/CC2D1b and Lgd2/CC2D1a. We will then discuss how Notch is activated in the absence of *lgd* function in *Drosophila* and ask whether there is evidence that a similar ligand-independent activation of the Notch pathway can also happen in mammals if the orthologs are inactivated.

Keywords

Notch signalling · Lgd · Endosomal pathway · CC2D1A · CC2D1B · ESCRT · Shrub/CHMP4 · Aki · FREUD-1 · FREUD-2 · TAPE

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Introduction

The Notch pathway is one of a handful evolutionary conserved signalling systems that mediate communication among cells during development and tissue homeostasis in all metazoans. At its core, it is a simple pathway that consists of three core components, the ligands, the Notch receptor and a nuclear factor of the CSL family (an acronym for *C*BF-1/RBPJ-κ of *Homo sapiens*/*Mus musculus*, *S*uppressor of Hairless of *Drosophila melanogaster* and *L*ag-1 of *Caenorhabditis elegans*) (e.g. reviewed in Kovall et al. (2017)). In *Drosophila*, in which the pathway was discovered, two ligands, termed Delta (Dl) and Serrate (Ser), a single Notch receptor and one CSL factor, termed Suppressor of Hairless (Su(H)), exist. Notch ligands are transmembrane proteins, thus reaching neighbouring cells only, making Notch signalling the main pathway to mediate short-range communication. Signal transduction is initiated by binding of the ligand to Notch, which elicits two proteolytic cleavages (for a simple overview, see Fig. 3.1). The result is the release of the intracellular domain of Notch (NICD) into the cytosol and its subsequent transport into the nucleus. There, it associates with CSL and co-factors, such as Mastermind (Mam) to initiate transcription of the target genes. Thus, in essence Notch is a transcriptional regulator initially tethered to the membrane as a surface receptor that is released upon the presence of a signal protein.

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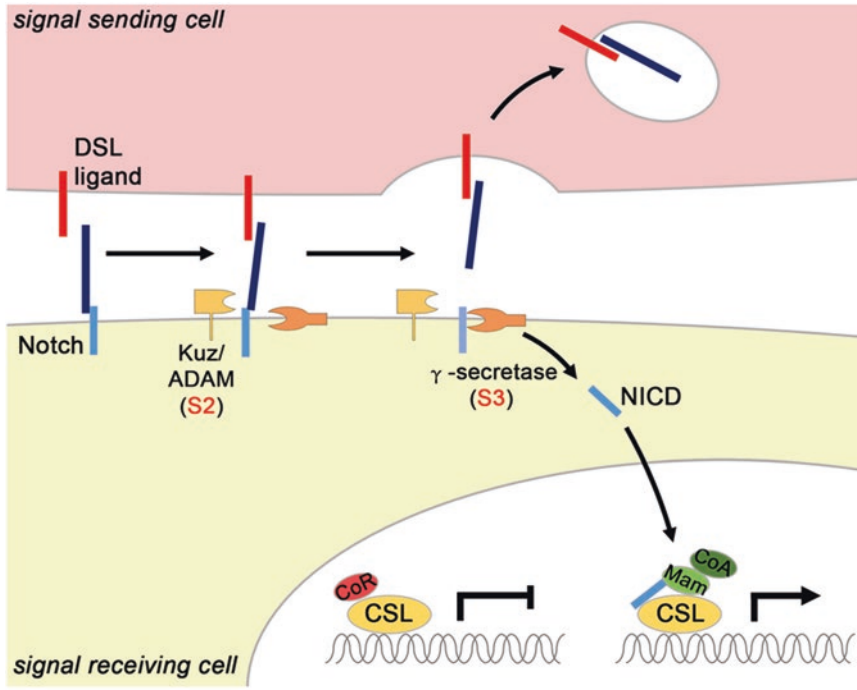

Fig. 3.1 A simplified view of the Notch signalling pathway. The binding of a DSL ligand to Notch and its subsequent endocytosis elicits a conformational change in Notch. This change releases Notch from autoinhibition via its NRR domain and allows the S2 cleavage via Kuz/ADAM10. The resulting intermediate (NEXT) is immediately cleaved by the γ-secretase complex to release NICD into the cytosol. NICD travels to the nucleus to assemble a transcriptional activator complex around CSL to activate the target genes. In the absence of a Notch activity, CSL associates with transcriptional co-repressors to actively suppress the expression of the target genes

Notch receptors are heterodimers whose two parts arise from the cleavage of a long precursor in the Golgi apparatus by a furin protease (reviewed in Arnett et al. (2018)). This S1 cleavage occurs in the extracellular part, located shortly behind the transmembrane domain. The two emerging parts are joint together via a salt bridge between Ca^{2+} and cysteine in the heterodimerisation domain (HD).

The mammalian genome contains four Notch receptors, five ligands and one CSL factor. Three orthologs of Dl exist, termed Dl-like (DLL) 1, 3 and 4, and two of Ser, termed Jagged (JAG) 1 and 2.

Notch receptors are large type I transmembrane proteins that, in addition to the HD, share a number of motifs in their extra- and intracellular domains (abbreviated ECD and ICD, respectively). The ECD contains 29–36 repeats of the EGF-like motif followed by the negative regulatory region (NRR), which is crucial for autoinhibition of the receptor in the absence of ligands. The NRR comprises the HD and three adjacent LIN12/Notch repeats (LNR), which are wrapped around the cleavage site for the ADAM10 protease performing the first of two activating cleavages, termed S2. In the autoinhibited state, the S2 cleavage site is obscured by the LNRs and becomes accessible during activation by a conformational change elicited by ligand pulling force. This pulling force is created through endocytosis of the ligand into the signal-emitting cell (Fig. 3.1). The resulting membrane-inserted fragment is termed Notch extracellular truncation (NEXT), which is immediately cleaved in its transmembrane domain by the γ-secretase complex (S3 cleavage). Importantly for this chapter, the S3 cleavage is relatively unspecific, since it has been shown that γ-secretase cleaves all Notch variants with a small ECD (below 150 amino

acids), even if the ECD or the transmembrane domain is replaced by an unrelated peptide sequence (Struhl and Adachi 2000). Hence, also NEXT-like variants will be efficiently cleaved by γ-secretase to release NICD. The S3 cleavage can potentially occur at the plasma membrane as well as in endosomal compartments or the lysosome, as γ-secretase is present in all these cell compartments (Vaccari et al. 2008; Pasternak et al. 2003).

All canonical Notch ligands belong to the Delta/Serrate/Lag2 (DSL) family which share several structural features. The ECD contains a module at the N-terminus of Notch ligands (MNNL) and Delta/Serrate/LAG-2 (DSL) domain in their N-terminus followed by a stretch of multiple repeats of the EGF-like motifs (Arnett et al. 2018). They possess relatively short ICDs, which appear to be unstructured and share no obvious sequence similarity, except short stretches that serve as binding epitopes for two E3 ligases (Weinmaster and Fischer 2011). These E3 ligases, termed Mindbomb1 (Mib1) and Neuralized (Neur) in *Drosophila,* bind and ubiquitylate the ICDs of the ligands at distinct lysins (Ks). Orthologs exist in mammals termed Mindbomb1 (MIB1) and Neuralized-like 1 and 2 (Neurl1, 2), respectively. The ubiquitylation (ubi) is thought to elicit the endocytosis of the ligands that in turn creates the pulling force required to release Notch from autoinhibition. Experiments in *Drosophila* showed that, in contrast to Ser, Dl can also weakly signal in the absence of ubi by the E3 ligases (Berndt et al. 2017). Remarkably, the weak ubi-independent signal of Dl appears to be sufficient for developmental processes, such as neurogenesis, to occur without gross defects. Recent work indicates that Neur activates Dl mainly in an ubi-independent manner (Berndt et al. 2017).

MIB1 simultaneously binds to two epitopes in the ICD of JAG1 and Dl, termed N- and C-Box, via its MZM and REP domains, respectively (Daskalaki et al. 2011; McMillan et al. 2015). The binding is a prerequisite for ubi and full activity of the ligand. Neur binds to a separate short stretch in the ICD of Dl and Ser, closer to the membrane with the consensus sequence NxxN (Fontana and Posakony 2009).

The target genes activated by the Notch pathway are tissue and context specific, and only few targets can be classified as general targets, among them genes of the HES/HER family of transcription factors; Nrarp, a negative regulator of the NICD/CSL transcription complex; and the transcriptional regulator Myc. Myc is a powerful common positive regulator of cell proliferation and an important driver of malignant transformation in several Notch-induced cancers (e.g. see Aster et al. (2017) for more details).

Previous work established that the Notch pathway can be activated also in more unconventional manners in mammals, e.g. by non-canonical ligands or in a ligand-independent manner upon its travel through the endosomal pathway. Examples of non-canonical ligands are MAGP1/2, DLK1 and YB1 (see Siebel and Lendahl (2017) and citations therein).

Endosomal Trafficking of Notch

Despite its role during ligand-dependent signalling, the endosomal pathway controls Notch activity by (i) degrading nonactivated Notch receptor, (ii) regulating receptor abundance on the cell surface and (iii) also in the generation of ligand-independent Notch signal. For an excellent overview of the pathway, the reader is referred to Huotari and Helenius (2011). An overview of the endosomal pathway and the journey of Notch therein is shown in Fig. 3.2. Notch is constantly endocytosed from the cell surface in a ligand-independent manner in all model systems (Chastagner et al. 2017; Jekely and Rorth 2003; Vaccari et al. 2008; Windler and Bilder 2010; Schnute et al. 2018). The presence at the plasma membrane is therefore relatively short, e.g. the resident time at the plasma membrane of *Drosophila* imaginal disc cells is shorter than the time required for mCherry maturation, which is between 40 and 80 minutes (Couturier et al. 2014). In uptake experiments with an antibody that binds the ECD of Notch, Notch localises in early endosomes (EEs) already after 5 min and is completely degraded after 5 h (Vaccari et al. 2008; Windler and Bilder 2010). In mammalian

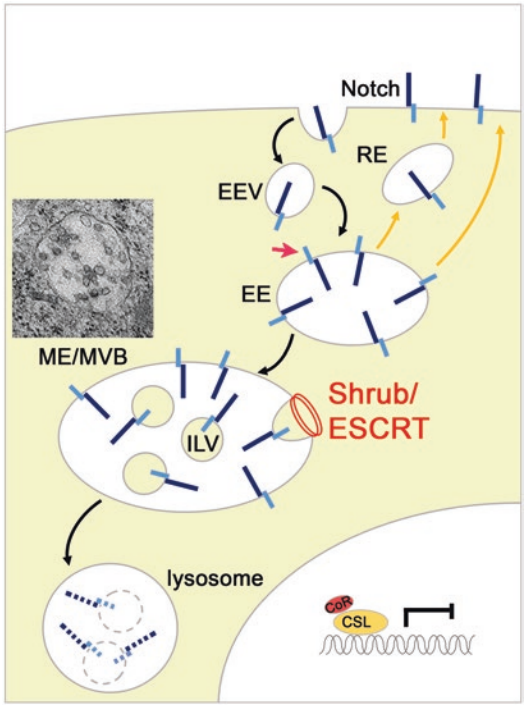

Fig. 3.2 Degradation of the Notch receptor by the endosomal pathway. Upon endocytosis, Notch is incorporated in early endosomal vesicles (EEVs). These EEVs undergo homotopic fusion to form an early endosome (EE) or fuse with already existing EEs. In the EE, Notch can return to the plasma membrane through a RME8-dependent pathway, which might include the passing through the recycling endosome (RE). However, the majority of Notch remains in the endosome, which matures and fuses with the lysosome where its content is degraded by the activity of acidic hydrolases. In the EE, the ICD of Notch is still in contact with the cytosol (red arrow). In order to get completely degraded, the ICD must be transferred into the lumen of the maturing endosome (ME). This is achieved by the action of the ESCRT machinery, whose central factor is Shrub/CHMP4. It mediates the abscission of Notch−/cargo-containing intraluminal vesicles (ILVs) into the lumen of the ME. As a result, the ME accumulates ILV and is recognisable as multi-vesicular bodies (MVBs) in the electron microscope (see insert). After incorporation of the cargo into ILVs, the ME fuses with the lysosome and the content is degraded

cells, pulse-chase experiments revealed that Notch1 appears in (not further specified) endosomes after 30 min and is degraded already after 60–90 min (Chastagner et al. 2008).

Endocytosis of Notch is thought to be initiated by ubi of its ICD, performed by several E3 ligases, such as Deltex (Dx) and members of the NEDD4 family, e.g. Suppressor of Deltex (Su(dx)) (Itch in mammals) and neural precursor cell-expressed, developmentally downregulated 4 (NEDD4) (reviewed in Schnute et al. (2018)). However, the requirement of ubi for endocytosis has not been rigorously tested. Indeed, it appears that Su(dx), as well as Dx, can elicit endocytosis of Notch in an ubi-independent manner, at least if over-expressed (Matsuno et al. 2002; Shimizu et al. 2014). Hence, it is possible that they act as adapters physically linking Notch to the endocytosis core machinery, e.g. AP-2 and clathrin. Indeed, Dx is suggested to form a complex with the nonvisual ß-arrestin Kurz, which binds to AP-2 and clathrin in *Drosophila* (Mukherjee et al. 2005).

As a result of endocytosis, Notch-containing early endosomal vesicles either fuse with each other to generate a new EE or fuse with already existing EEs (Fig. 3.2). In EE, Notch can return to the plasma membrane via a poorly characterised Rme8-/Rab4-dependent recycling pathway, but the majority is destined to be degraded in the lysosome. Therefore, Notch remains in the EE, which matures and eventually fuses with the lysosome where the luminal content of the matured endosome is degraded. In the EE, Notch is inserted in the limiting membrane (LM), meaning its ICD is still in contact with the cytosol (Fig. 3.2, red arrow). However, in order to get completely degraded, the ICD must be translocated into the lumen of the maturing endosome (ME). This is achieved by concentrating Notch (as well as other transmembrane cargo) at one spot of the LM, which is subsequently abscised into the lumen to form an intraluminal vesicle (ILV, Fig. 3.2). For the integration into ILVs, the cargo must be ubiquitylated. During maturation the endosome accumulates ILVs and is recognised as a multi-vesicular body (MVB) in the electron microscope (Fig. 3.2, insert). The abscission of ILVs is a complex process for which the eukaryotic cell uses a set of protein complexes collectively known as the endosomal sorting complex required for transport (ESCRT) machinery (Hurley 2015). The machinery consists of five in sequence acting complexes, termed ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and

Vps4. ESCRT-0–II are stable complexes, which are recruited to the LM of the EE from the cytosol, whereas ESCRT-III assembles only on the LM from its monomeric cytosolic components. ESCRT-0–II recognise cargo via its ubiquitin label and concentrate it at a certain spot. ESCRT-II is also required to initiate the assembly of ESCRT-III, which is responsible for membrane abscission. For this purpose, it forms a polymer, consisting of Shrub or CHMP4 in *Drosophila* and mammals, respectively. The polymer is capped by Vps2/CHMP2 and Vps24/CHMP3. The polymerisation of Shrub/CHMP4 is initiated by Vps20/CHMP6. All four ESCRT-III core components are members of the CHMP family and share the same basic conformation. Shrub/CHMP4 appears to assume two different conformations. In the cytosol, it exists in the closed conformation in which the negatively charged C-terminus folds back to contact the positively charged N-terminus that forms a helical bundle. At the LM it opens up to assume an extended helical hairpin conformation that allows polymerisation. The details of polymerisation are not understood, but Shrub in its open form has two complementary electrostatic surfaces that promote the association into a staggered array (McMillan et al. 2016; Tang et al. 2015) (Fig. 3.4d). The electrostatic surfaces are conserved among all Shrub orthologs in metazoans, suggesting that the mode of polymerisation is evolutionary conserved. Polymerisation of ESCRT-III is dynamic, and an assembled polymer is quickly disassembled by the AAA-ATPase complex Vps4. This disassembly is as crucial as assembly of ESCRT-III for the abscission to occur (Adell et al. 2017). After incorporation of Notch and other cargo into ILVs, the ME eventually fuses with the lysosome, and the luminal cargo is digested by the present activated acidic hydrolases (Fig. 3.2). As expected, the analysis of ESCRT-mutants in the electron microscope documented a severe distortion of the formation of ILVs and a dramatic enlargement of the MEs in ESCRT mutant cells (Stuffers et al. 2009).

ESCRT-III together with Vps4 as a membrane abscission apparatus is used in many other processes where membrane is abscised away from the cytosol, such as membrane repair, cell division and axon pruning. Moreover, it is hijacked by many enveloped viruses to exist in the infected cell, such as HIV and the Ebola virus (see Vietri et al. (2020)).

Lethal (2) Giant Discs (lgd)

lgd is classified as a tumour suppressor gene in *Drosophila*, whose loss of function (lof) causes the formation of giant imaginal discs due to over-proliferation of the disc cells ((Bryant and Schubiger 1971, Buratovich and Bryant 1997), Fig. 3.3a, b'). *lgd* mutants die during the early pupal phase, probably due to the inability of the overgrown imaginal discs to correctly fuse with each other to form the body wall of the imago. Importantly, the epithelial organisation of the discs is not affected, indicating that *lgd* belongs to the class of hyperplasia causing tumour suppressor genes (hyperplastic class). Analysis of *lgd* mutant wing imaginal discs revealed that Notch target genes are ectopically activated in the wing primordia and that the over-proliferation is suppressed by loss of Notch signalling ((Klein 2003), Fig. 3.3a, b'). These findings indicate that the Notch pathway is either over-activated or ectopically activated in *lgd* mutants. Indeed, further analysis showed that the pathway is ectopically activated in all disc cells in a ligand-independent manner (Childress et al. 2006; Gallagher and Knoblich 2006; Jaekel and Klein 2006). In addition, the ectopic activation in *lgd* mutant cells is independent on Kuzbanian (Kuz), the ADAM10 ortholog in flies performing the S2 cleavage, but dependent on the activity of the S3 performing γ-secretase (Jaekel and Klein 2006; Schneider et al. 2012). Altogether, the results indicated that *lgd* lof causes uncontrolled ligand-independent activation of Notch in all imaginal disc cells in a cell autonomous manner. This holds true also for cells of the follicle epithelium of the ovary, suggesting that the activation of Notch is a general consequence of loss of *lgd* function in *Drosophila* (Morawa et al. 2015).

Cells mutant for *lgd* contain enlarged Notch-positive MEs, indicating that Lgd is required during endosomal trafficking of Notch ((Schneider et al. 2012), Fig. 3.3d, d'). The ectopic activation

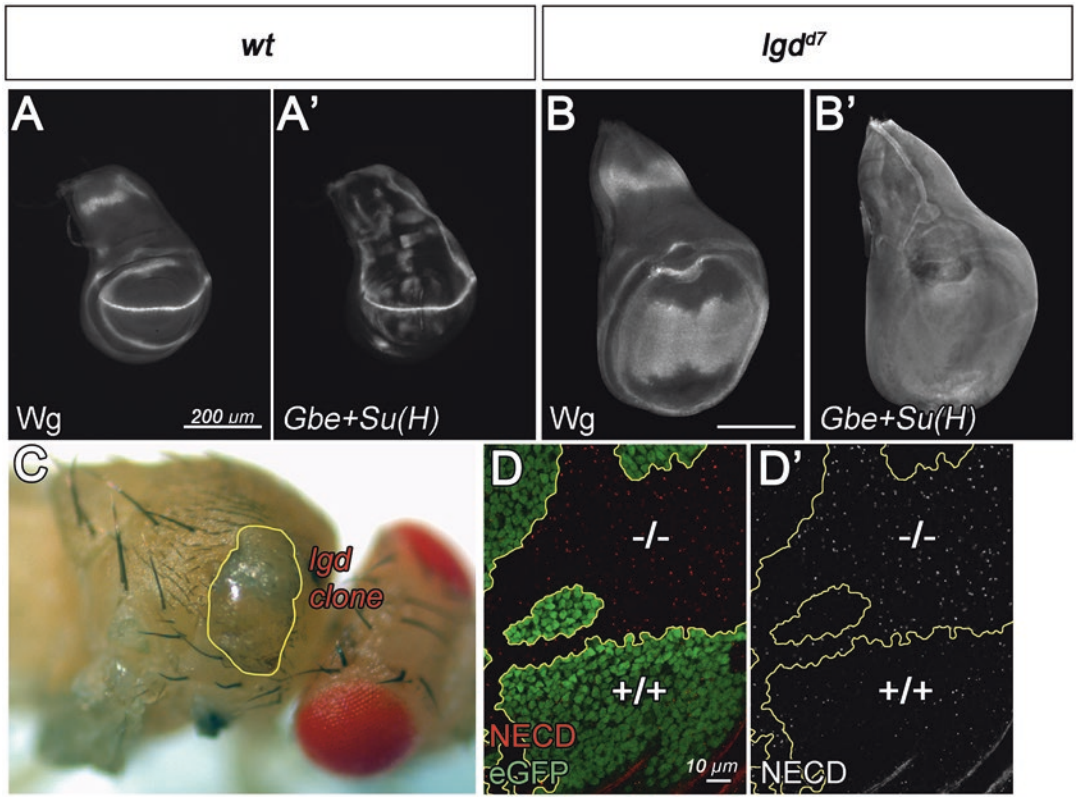

Fig. 3.3 The phenotype of lof of *lgd* in *Drosophila*. (**a**, **a**') Expression of the endogenous target gene Wg (A) and the Notch activity reporter construct Gbe + Su(H) (**a**') in wild-type wing imaginal discs. (**b**, **b**′) Expression of the marker in *lgd* mutant discs. The expression of the tissue-specific target Wg is dramatically expanded, while that of the more sensitive Gbe + Su(H) occurs in all disc cells, indicating ubiquitous activation of the Notch pathway in the disc. (**d**, **d**') Distribution of Notch in discs bearing *lgd* mutant cell clones (−/−). The clones are labelled by the absence of GFP; the distribution of Notch is revealed by antibody staining. The optical focus is within the cells and reveals that Notch is distributed in punctae in the cytosol. These punctae are MEs. The comparison shows that the mutant cells contain more and larger Notch-positive MEs

of Notch is suppressed in *lgd* mutants if the function of either *hrs*, which encodes a component of ESCRT-0, or of *rab7*, the organiser of fusion with the lysosome, is abolished (Schneider et al. 2012; Gallagher and Knoblich 2006). These findings indicate that the endosomal defect is the cause for the activation of the pathway in *lgd* mutants.

The ultrastructural analysis revealed that the mutant cells contain all size classes of MEs of wild-type cells, but in addition also larger size classes (Schneider et al. 2012). This phenotype can be explained by an increased lifetime of MEs in the mutant cells, which would allow more homotopic fusions between MEs to occur. In support of this interpretation is the finding that the degradation of cargo, such as Notch, is also delayed in *lgd* cells, suggesting a longer lifetime of the MEs (Schneider et al. 2012).

A genetic screen identified *shrub* as a locus that is in functional relationship with *lgd* (Troost et al. 2012). Genetic interaction studies suggest that *lgd* is required for the full activity of *shrub*. In the absence of *lgd* function, the activity of shrub appears to be reduced, but not abolished. This assumption is based on the observation that *lgd* mutants die at an earlier time point if one copy of *shrub* is removed (genotype *lgd* +/*lgd shrub*), although *shrub*/+ flies are vital and display no pattern defects. Hence, the shift in the time point of death of the *lgd* +/*lgd shrub* flies

suggests that the activity of Shrub is lowered in *lgd* mutants to a degree that does not allow further reduction, e.g. brought about by removal of one copy of *shrub*. Altogether, the analysis established Lgd as a positive regulator of the activity of ESCRT-III.

Similar to *lgd*, lof of *shrub* or other genes encoding elements of ESCRT-I, ESCRT-ll and ESCRT-III cause ectopic ligand-independent activation of the Notch pathway (Thompson et al. 2005; Vaccari and Bilder 2005; Vaccari et al. 2009). However, lof of ESCRT additionally prolongs signalling via other pathways, such as the BMP/Dpp and Wg/Wnt pathways (Thompson et al. 2005). A similar prolongation of signalling via the BMP/Dpp pathway has been observed upon lof of *lgd* only in the germline and the follicle epithelium of the ovary (Morawa et al. 2015). The prolonged signalling causes the formation of supernumerary stem cells and the induction of one additional round of cell division of the cyst cells. The *lgd* mutant phenotype resembles that observed in the ovary of *shrub* heterozygous flies, indicating that also in this case Lgd is functionally connected to Shrub (Morawa et al. 2015).

A further consequence of the loss of ESCRT function is the loss of epithelial polarity that in combination with the cell over-proliferation leads to the formation of a large multilayer of undifferentiated cells (neoplastic phenotype), instead of a well-patterned epithelial monolayer (Vaccari et al. 2009). These additional phenotypes of ESCRT mutants are not observed in *lgd* mutants, although Lgd is a positive regulator of Shrub. The discrepancy in the phenotypes is not understood. A possible explanation is that the reduction, but not complete loss of *shrub* activity, caused by the loss of *lgd* function only causes a hyperplastic phenotype, while the complete lof of *shrub* causes the more drastic neoplastic phenotype.

The Molecular Function of Lgd

lgd encodes a protein of 816 amino acids that contains 4 repeats of the DM14 domain, followed by a linker region and a C2 domain ((Childress et al. 2006; Gallagher and Knoblich 2006; Jaekel and Klein 2006), Fig. 3.4a). Orthologs exist in all metazoans with similar domain organisation. Mammals have two variants in their genomes, termed LGD1 and LGD2. LGD1 and LGD2 have alternative names, which partly reflect the different functions found in experiments: LGD1 is also called coiled-coil and C2 domain-containing protein 1B (CC2D1B) and FRE-binding protein, five repressor element under dual repression-binding protein-2 (FREUD-2), whereas LGD2 is also termed CC2D1A, Akt kinase-interacting protein 1 (Aki1), TBK-associated protein in endolysosomes (TAPE) and FREUD-1 (Matsuda et al. 2003; Ou et al. 2003; Hadjighassem et al. 2009; Nakamura et al. 2008; Chang et al. 2011). For reasons of simplicity, we will use a double name Lgd/CC2D1 in the following.

The unique hallmark of the Lgd family is the helical hairpin forming DM14 domain ((Childress et al. 2006, Gallagher and Knoblich 2006, Jaekel and Klein 2006, McMillan et al. 2017) Fig. 3.4c). All family members of metazoans have four tandem repeats of this domain. Pull-down experiments indicated that the DM14 domain-containing region is required for the direct physical interaction with Shrub, revealing a molecular basis for the functional relationship of both genes found in the genetic analysis (McMillan et al. 2017). Sequence comparison of the four DM14 domains revealed that, in each ortholog, the odd-numbered domains (DM14–1 and DM14–3) are more similar to each other than to the even-numbered ones (DM14–2 and DM14–4). Likewise, the even-numbered domains are more related to each other than to the odd-numbered ones. The odd-numbered DM14 domains have an extended positively charged surface (KARR motif) that is absent in the even-numbered domains (Fig. 3.3b). Lgd binds directly to the negative electrostatic surface of Shrub via the positive surface of the odd-numbered domains (Fig.3.3d (McMillan et al. 2017)). The negative surface of Shrub is also required for its homo-polymerisation at the LM of the ME, suggesting that binding of Lgd to Shrub and polymerisation of Shrub are mutually exclusive events (Fig. 3.4d).

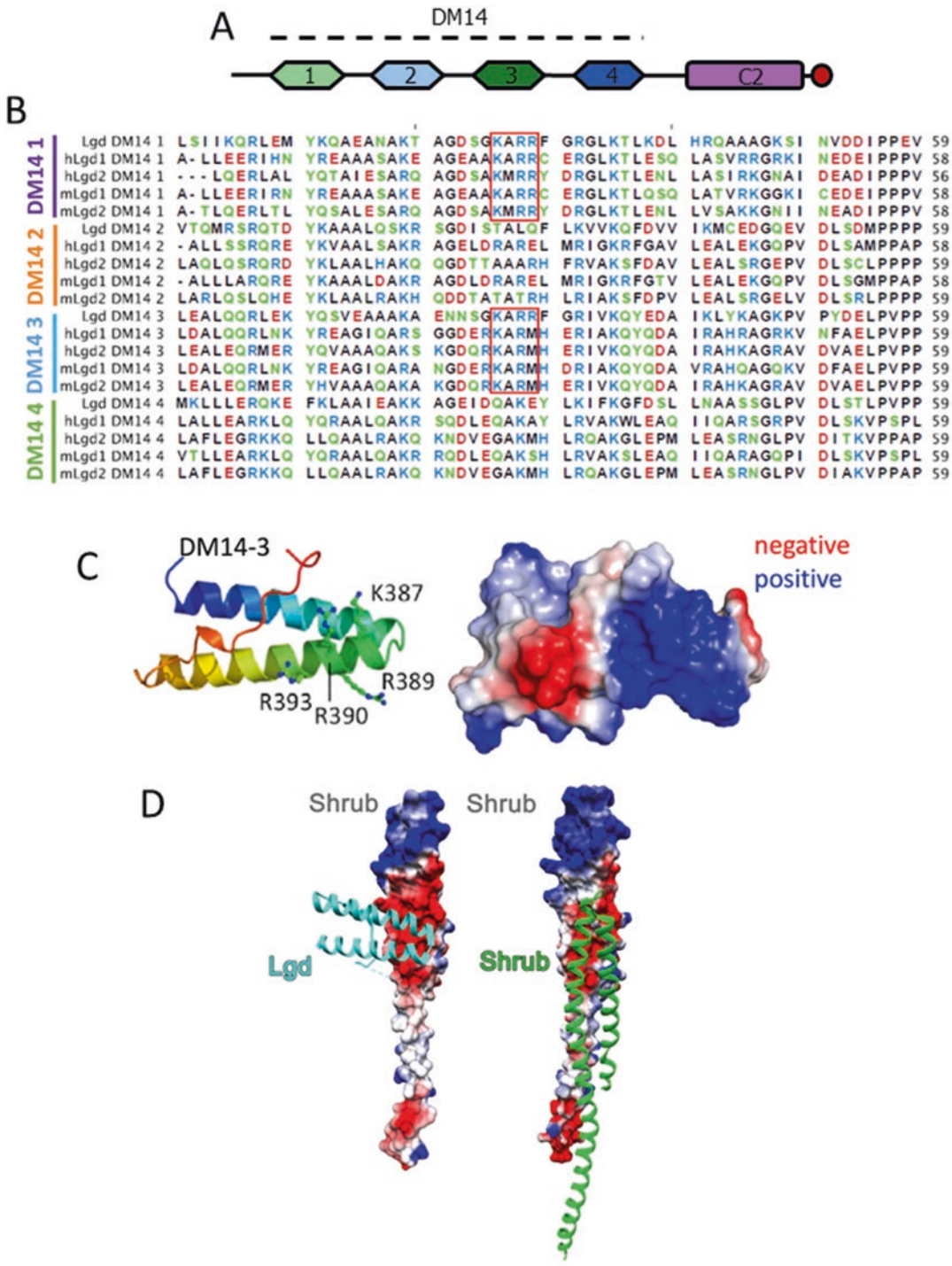

Fig. 3.4 The molecular characterisation of Lgd/CC2d1 proteins. (**a**) Domain architecture of Lgd/CC2D1a. It consists of four repeats of the DM14 domain followed by a linker region that connects the DM14 domains with a C2 domain at the C-terminus. (**b**) Sequence comparisons of the DM14 domains of Lgd with the orthologs of mouse and human. It reveals that the odd-numbered domains contain an extended positive surface (KARR motif). (**c**) Atomic structure of the third DM14 domain of Lgd. It is a helical hairpin with a positive charged surface patch created by the KARR motif (blue). (**d**) The positive patch binds to the negative surface of Shrub. This surface is also required for the polymerisation of Shrub. Hence, Lgd binding to Shrub and Shrub polymerisation are mutually exclusive

Strangely, although the even-numbered DM14 domains are present in all orthologs, they appear to be dispensable for function in *Drosophila*, indicated by the finding that Lgd variants with only one odd-numbered domain (e.g. DM14–3) can fully rescue *lgd* mutants, while variants with only even-numbered ones (e.g. DM14–4) failed to do so (McMillan et al. 2017). In further support of this conclusion, DM14–4 does not bind to Shrub in vitro (McMillan et al. 2017). The loss of its C2 domain results in a mis-localisation of Lgd into the nucleus and a loss of its activity. Hence, the C2 domain is required for the correct subcellular localisation of Lgd in the cytosol. Additionally, it contributes to the stability of Lgd (Troost et al. 2012).

Experiments with tagged versions and anti-Lgd antibody staining revealed that Lgd is diffusely distributed in the cytosol (Childress et al. 2006, Gallagher and Knoblich 2006, Jaekel and Klein 2006). Combined with the fact that Lgd binding to Shrub and Shrub polymerisation is mutually exclusive, this finding suggests that Lgd binds to the monomeric cytosolic closed form of Shrub. It is assumed that adopting the closed conformation is essential to prevent inappropriate polymerisation in the cytosol. Thus, a possible way how Lgd might act is that by binding it helps Shrub to adopt or stay in the closed conformation.

Activation of Notch During Endosomal Trafficking in *lgd* Mutants

Lof of *shrub* or *lgd* results in activation of Notch during endosome maturation. The major task of Shrub in this process is the formation of ILVs and in its absence this process is impaired. A consequence of the failure of ILV formation is that Notch and other cargo remains in the LM of the ME. The ICD of Notch in the LM protrudes in the cytosol, even after fusion with the lysosome. Somehow the ICD of Notch must be released from the LM of the lysosome. A realistic scenario how this occurs is that the active acidic hydrolases of the lysosomal lumen degrade the ECD of Notch, thereby creating a NEXT-like variant inserted in the LM that is cleaved by γ-secretase. γ-Secretase at the lysosome is very abundant and active (Pasternak et al. 2003). Alternatively, or in addition, the acidic environment and the occurring export of ions, especially Ca^{2+}, from the lumen of the ME, might be sufficient to severe the salt bridge in the HD of Notch and thereby achieve the shedding of the ecto-domain of Notch (reviewed in Scott and Gruenberg (2010)). In any case, the result is an alternative ecto-domain shedding of Notch as it also occurs during ligand-dependent activation. A similar model has been suggested also for the activation of Notch in *lgd* mutants, but a few differences have to be taken into account (Fig. 3.5). If *shrub* activity is reduced in *lgd* mutants, one possible effect would be a failure of ILV formation. However, *lgd* mutant cells still contain MVBs, indicating that ILV formation still occurs (Schneider et al. 2012). Thus, in principle Notch can be incorporated into ILVs and thereby removed from the LM in Lgd cells. It is possible that the rate of ILV formation is reduced, thereby allowing a fraction of Notch to remain on the LM. However, it has not been determined so far, whether *lgd* mutant MVBs contain fewer ILVs. Nevertheless, elegant recent work by the Schweisguth lab suggests that indeed a fraction of Notch escapes the incorporation into ILVs in *lgd* mutants (Couturier et al. 2014). It is this fraction which is thought to be activated as described upon fusion of the ME with the lysosome (Fig. 3.5 (Schneider et al. 2012)). Further results provide support for the suggested model of Notch activation in *lgd* mutant cells: the activation (1) depends on the fusion of the ME with the lysosome and (2) requires the activity of the V-ATPase that acidifies the lumen of the ME (Schneider et al. 2012; Troost et al. 2012). The acidification is required for the acidic lysosomal hydrolases to be activated. Although the proposed model of activation is plausible, it is not proven. For example, it has not been shown whether Notch that remains at the LM of the lysosome is really activated in a ligand-independent manner.

It is noteworthy to mention here that the endosomal activation of Notch is required for proper development in *Drosophila*. It has been shown

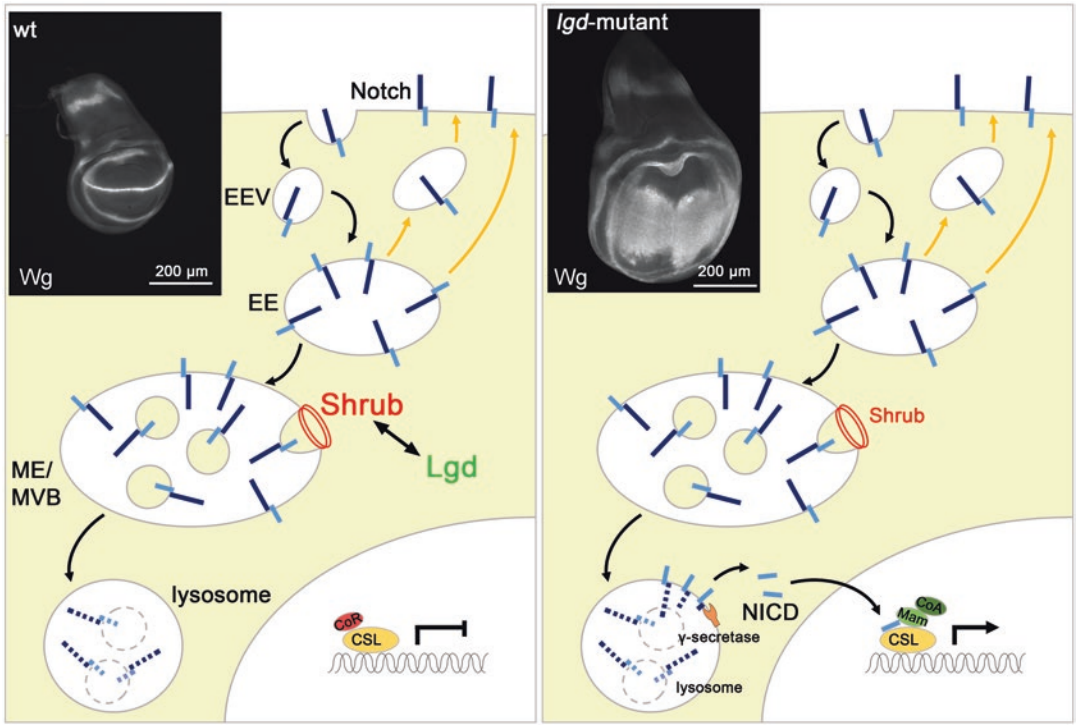

Fig. 3.5 Model of activation of Notch in *lgd* mutant cells. In *lgd* mutant cells, Notch is normally endocytosed and transported to the EE. However, due to the reduction of the activity of Shrub, a fraction of Notch escapes the incorporation into ILVs. Upon fusion with the lysosome, the ECD of this fraction is removed by the activity of the acidic hydrolases. Alternatively, or in concert, the acidic environment of the lysosomal lumen and the lack of Ca2+ induce the separation of the ECD from the remainder of Notch. In any case, the result of this ligand-independent ecto-domain shedding is a NEXT-like intermediate that is cleaved by ɣ-secretase to release NICD into the cytosol. NICD travels to the nucleus where it activates the expression of the target genes in an activator complex with CSL and co-factors

that an additional function of Su(dx) and Dx is to regulate the amount of Notch incorporated into ILVs. The Su(dx)/Dx mechanism assures a basal activity of Notch signalling required for development (Shimizu et al. 2014; Wilkin et al. 2008).

Function of Lgd in Mammals

So far, the information about the function of Lgd in mammals is confusing and points to a variety of functions. Lof of Lgd2/CC2D1a in mice results in postnatal death due to a brain defect that causes a failure in breathing (Al-Tawashi et al. 2012; Drusenheimer et al. 2015; Chen et al. 2012; Zhao et al. 2010). The conditional KO of Lgd2/Cc2D1a in the forebrain causes a variety of behavioural deficits that are cognitive and social impairment, anxiety, hyperactivity and repetitive behaviours (Oaks et al. 2017). Interestingly, lof of LGD2/CC2D1A in humans is not lethal after birth, but causes mental retardation and autism spectrum disorder (Basel-Vanagaite et al. 2006; Manzini et al. 2014). A likely cause of these pathologic traits is a reduction in dendritic complexity observed in ex vivo culture assays (Manzini et al. 2014).

In contrast, lof of Lgd1/CC2D1B has no obvious consequences for development or life span, and homozygous mutant mice can be maintained as a strain (Drusenheimer et al. 2015). The lack of phenotype of *Lgd1* mutants can be a result of an extensive functional identity/compensation/redundancy among both LGDs in mammals.

Indeed, this appears to be the case: both genes are similarly expressed in all tissues of mouse tested (Drusenheimer et al. 2015). Moreover, *Lgd1 Lgd2* double mutant mice die early in development, before E11.5 (Zamarbide et al. 2018). Since single mutants either die after birth with no gross defects (Lgd2/CC2D1a) or are viable (Lgd1/CC2D1b), this synergistic phenotype indicates an extensive functional redundancy among the Lgd/CC2D1 paralogs. Unfortunately, neither the double mutant embryos nor double mutant MEFs have been analysed so far.

Although the two Lgd paralogs share a functional redundancy, they also differ in some aspects, which is already indicated by the difference in lethality. The difference is also manifested in other phenotypic aspects: lof of LGD2/CC2D1A in the forebrain causes an array of cognitive and social defects, anxiety and hyperactivity, while the lof of LGD1/CC2D1B leads only to cognitive deficits (Zamarbide et al. 2018).

Several experiments revealed that the molecular relationship between Lgd and Shrub is conserved in mammals as well. Rescue experiments in *Drosophila* showed that LGD1/CC2D1B and LGD2/CC2D1A can rescue *lgd* mutant flies, albeit to different degree (Drusenheimer et al. 2015). While LGD1, which is more similar in sequence to Lgd than LGD2, rescues completely, the recue by LGD2 is only partial, even if present in two copies in the genome. The rescue of *lgd* mutants by the mammalian orthologs is further proof that both mammalian paralogs have an at least partially redundant molecular function, which is shared by Lgd of *Drosophila*. The rescue activity of LGD2 in flies is dependent on the function of *shrub*, suggesting that also the functional relationship with Shrub is conserved (Drusenheimer et al. 2015). In agreement, it has been shown that both mammalian Lgd orthologs bind to the three Shrub orthologs in mammals CHMP4B, CHMP4A and, weaker, CHMP4C (Drusenheimer et al. 2015; Usami et al. 2012; Tsang et al. 2006). The binding to CHMP4B appears to be mediated largely via DM14–3. Hence, the intimate relationship between Lgd and Shrub appears to be conserved in evolution.

Similar to *Drosophila*, in vitro and in vivo studies also point to a role of Lgd1 and Lgd2 in the regulation of the function of the ESCRT machinery in mammals. LGD2/CC2D1a has been found as an endosomal protein in a multiparametric survey of endocytosis, as a factor that negatively affects endosomal trafficking in cell culture cells (Collinet et al. 2010). We found that (1) the deficiency of *Lgd2* results in an enlargement of MEs, (2) Lgd2 and Chmp4b interact with each other in living cells and (3) both Lgds cycle together with CHMP4B between the cytosol and the LM of the ME (Drusenheimer et al. 2015).

It appears that the LGDs are also required for the regulation of several signalling pathways in mammals, ranging from Toll, over the EGFR, to the nuclear factor kB, to PDK1/Akt, to cAMP/PKA, to bone morphogenetic protein and PKA signalling (Zhao et al. 2010; Rogaeva et al. 2007; Ou et al. 2003; Hadjighassem et al. 2009; Chen et al. 2012; Chang et al. 2011). At least in the case of TLR and EGFR signalling, the effects are linked to a defect in ESCRT function or at least to a malfunction of endosomes (Chang et al. 2011; Deshar et al. 2016). Whether a malfunction of the endosomal pathway is causative also for the effects on the activity of the other pathways remains to be determined.

Besides the conserved function in endocytosis, LGDs in mammals appear to have additional functions so far not identified in *Drosophila* and independent of endosome trafficking. It has recently been shown that LGD1/CC2D1B is required for the correct timing of nuclear envelope closure after cell division in concert with the Shrub ortholog CHMP4B (Ventimiglia et al. 2018). Lof of LGD1/CC2D1B results in defective envelope reformation and therefore causes an aberrant envelope morphology. Interestingly, this defect appears not to severely impact on vitality of LGD1/CC2D1B mutant mice. As mentioned above, mutant mice are healthy and fertile. A possible explanation for this puzzle is the incomplete penetrance of the envelope phenotype. It is not known whether also LGD2/CC2D1A is involved in envelope reformation and is responsible for this lack of penetrance.

The study by Ventimiglia et al. (2018) also revealed a second important aspect: they found that LGD1/CC2D1B interacts also with another CHMP family member, CHMP7, during envelope closure. In this case the fourth DM14 domain mediates the direct interaction between the proteins (Ventimiglia et al. 2018). Moreover, they found that Lgd2/CC2D1B can interact with CHMP2A. Hence, the LGDs can interact with several members of the CHMP family. The significance of these interactions might have been overlooked so far, since, like LGD, CHMP2A and CHMP7 regulate the activity of CHMP4B. Hence, the consequence of their inactivation is always the lof of CHMP4B. Interestingly, LGD2/CC2D1a appears not to interact with CHMP2A (Usami et al. 2012). Thus, there might be also differences among the LGDs in the capacity of binding CHMP family members.

For the more studied LGD2/CC2D1a, a variety of additional functions have been found which appear not to be associated with its endosomal function. It has been reported to regulate centrosome cohesion, and loss of its function causes formation of multipolar spindles during cell division and multinucleated cells in HeLa cell cultures (Nakamura et al. 2009). However, the phenotypes were not observed in MEFs obtained from Lgd2 mutant mice, suggesting that either a functional difference of Lgd2 between mice and humans or a differential requirement in diverse cell types exists (Drusenheimer et al. 2015). In another function, LGD2/CC2D1A appears to act as a scaffold protein that assembles complexes important in cAMP/PKA and PDK1/AKT signalling (Al-Tawashi et al. 2012; Nakamura et al. 2008). In the case of the PDK1/Akt1 module, LGD2/CC2D1a (here termed Aki/FREUD1) links the module specifically to the EGFR. Interestingly, also the interaction of LGD2 with PDK1 appears to be mediated by the fourth DM14 domain. The involvement in EGFR/Akt signalling raises the possibility that Lgd2/CC2D1A might be involved in cancer in a Notch-independent way in humans. Indeed, recent work suggests that it might be a potential therapeutic target in lung, ovarian and pancreatic cancer (Ohtsubo et al. 2014; Yamada et al. 2013; Kumar et al. 2019).

The most surprising function of LGD1/CC2D1A and LGD2/CC2D1B is the one as a transcriptional repressor that represses the expression of serotonin-1A auto-receptors. This finding provides a molecular link of both genes to depression and anxiety (Ou et al. 2003; Rogaeva et al. 2007; Hadjighassem et al. 2009; Vahid-Ansari et al. 2017). The DNA-binding activity of LGD2/CC2D1a has not been determined, but the linker region that connects DM14–4 with the C2 domain was predicted to fold in a HLH conformation typical for bHLH transcription factors (Ou et al. 2003). However, Freud-1 lacks a clear nuclear localisation sequence, and a sequence comparison between LGD2/CC2D1a (Freud-1) and several Lgd family members revealed that the putative HLH region is poorly conserved. Moreover, although not similar in sequence, the respective region in *Drosophila* Lgd adopts a similar helical hairpin conformation as the DM14 domains (Ventimiglia et al. 2018). Thus, it remains to be determined whether the proposed region has DNA-binding properties in all Lgds or is an additional DM14 domain.

Is Notch Activated in Mammals Upon Loss of LGD or ESCRT Function?

In *Drosophila* lof of *lgd* causes the uncontrolled ligand-independent activation of the Notch pathway due to a reduction of the endosomal function of the ESCRT machinery. This raises the obvious question whether the lof of LGD/CC2D1a or ESCRT function causes a similar activation of the Notch pathway. Uncontrolled activation of Notch has been observed as a cause of various cancers, among them T-cell leukaemia, such as T-ALL (Aster et al. 2017). In T-ALL the NRR of Notch1 is affected by mutations that results in a release of autoinhibition and activation of the receptor in a ligand-independent manner. However, this activation requires no defect in endosomal trafficking. It has been recently shown that Notch3 is activated apparently in a ligand-independent manner in breast cancer cells. Although this mechanism is not entirely understood, some results suggest that the activation

occurs at the plasma membrane (Choy et al. 2017). Whereas in these cases the endosomal involvement in ligand-independent Notch activation is not obvious, it appears to be a requirement for the ligand-independent activation of Notch in the immunological synapse (Steinbuck et al. 2018). Here, the activation of the T-cell receptor in combination with CD8 causes endocytosis of Notch and the activation of ADAM10 and ADAM17, which perform the S2 cleavage of Notch in the LM of the ME. Apparently, the luminal environment of the ME releases Notch from autoinhibition, allowing the S2 cleavage by the ADAMs. While in this case Notch is activated in the ME, it differs from ligand-independent activation in *ESCRT* or *lgd* mutant cells through the requirement of the S2 cleavage, by the ADAMs.

Altogether, these recent reports document that uncontrolled ligand-independent activation of the Notch pathway occurs also in mammals, even during endosomal trafficking, but evidence for its activation upon lof of *lgd* or ESCRT is scarce so far. Loss of ESCRT function in mouse causes lethality during early embryogenesis at a similar stage as in *Lgd1 Lgd2* double mutants (Wagner et al. 2003; Lee et al. 2007). In most cases the activity of Notch has not been monitored. In case of *CHMP5* lof, at least several pathways are activated, among them TGFß/BMP, RTK pathways that activate the ERK1/2 module, NFκB and also the TLR4 pathway (Shim et al. 2006). CHMP5, Vps60 in yeast, is a so-called accessory ESCRT protein, which is nonessential in yeast. It is required for full ESCRT-III/Vps4 function. In *Drosophila*, its lof causes a cold-sensitive phenotype that includes weak ectopic activation of the Notch pathway (Bäumers et al. 2019). Unfortunately, Notch signalling appears not to be tested in mouse CHMP5 mutants.

The mammalian intestinal epithelium is a rapidly renewing tissue where the differentiated cells are replaced within 2–5 days. Functionally distinct epithelial cells are constantly generated by resident stem cells in the crypt of each villus. Thus, matching intestinal stem cell (ISC) production and their timely differentiation has to be tightly controlled and accurate. Homeostasis is maintained by actively cycling ISC, so-called CBCs (crypt base columnar cells) expressing the Wnt pathway component Lgr5 (leucine-rich repeat-containing G-protein-coupled receptor 5) with basal location in the crypt (Barker et al. 2007). Self-renewal of CBCs is controlled through gradients of wingless-related integration site (WNT), Hedgehog (HH), bone morphogenetic protein (BMP) and Notch signalling pathways (Geissler and Zach 2012; Takebe et al. 2011). The lof of the Notch pathway results in the loss of the stem cells, while its ectopic activation causes a dramatic expansion of the stem cell compartment, indicating that it is an essential signal in the stem cell niche (). The pathway has a second function during differentiation of the precursor cells. When blocked, the stem and precursor cells differentiate into the secretory lineage causing a hyperplasia of mucus-secreting Goblet cells (GCs), while activation of the Notch pathways inhibits GC differentiation. Therefore, the density of GCs in the epithelium is a measure of Notch activity. This assay was used to test whether the KO of LGD1 or LGD2 in the gut epithelium causes changes in Goblet cell density. However, this was not the case, indicating that individual KO of either LGD ortholog does not significantly affect Notch signalling in the gut epithelium (Drusenheimer et al. 2015). Nevertheless, due to the extensive functional redundancy among the two Lgds, the final verdict is still out, as long as the double KO in gut cells is not analysed.

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Notch and Endometrial Cancer

4

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Abstract

The human endometrium is a unique, highly dynamic tissue that undergoes cyclic changes of cell proliferation, differentiation, and death. Endometrial cancer is the most common malignancy among women in developed countries. Importantly, the incidence of endometrial cancer is rising in high-income countries. Currently histological classification is used for subtyping of endometrial cancer, while ongoing research is evaluating markers for more accurate molecular classification. Evolutionary conserved Notch signaling pathway regulates diverse cellular processes such as proliferation, differentiation, and cell invasion. Accumulating evidence links aberrant Notch signaling with diseases such as hyperplasia and endometrial cancer. This chapter summarizes the current state of Notch signaling investigations in the endometrium, endometriosis, and endometrial cancer.

Keywords

Notch · Endometrium · Endometrial cancer · Endometriosis · Stem cells · Leptin

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Notch Signaling in the Normal Endometrium

The human endometrium is a unique, dynamic system that undergoes cyclic changes regulating cell proliferation, differentiation, and death during every menstrual cycle and pregnancy. Physiological changes that occur in fertile women are tightly regulated by hormones, specifically estrogen, progesterone, and chorionic gonadotropin (Banerjee and Fazleabas 2011; Maruyama and Yoshimura 2008). Therefore, it is not surprising that evolutionary conserved Notch pathway, due to its role in proliferation, differentiation, and angiogenesis, is actively involved in endometrium remodeling as well as in diseases such as hyperplasia and cancer.

Morphologically the endometrium is divided into functional and basal layers. The functional layer occupies two-thirds of the endometrial thickness and it is responsible for proliferation and secretion. During menstruations, the functional layer separates from the basal layer, while the basal layer serves as a base for endometrial regeneration and remains intact during menstruation. The endometrium is composed of different compartments: the luminal and the glandular epithelium, the stroma with stromal fibroblastic cells, and the vascular compartment (Diedrich et al. 2007; Ruiz-Alonso et al. 2012).

Notch is crucial for uterine development. In fact, it was shown that overexpression of

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NICD1 in mouse uterus leads to complete infertility, absence of uterine glands, and dysregulation of progesterone and estrogen signaling (Su et al. 2016). Mikhailik et al. have examined transcripts and demonstrated that Notch signaling is active in both the epithelial and stromal cells of the human endometrium. They have shown that all four *NOTCH* receptors are ubiquitously expressed in endometrial cells, whereas ligands *JAG1* and *DLL4* and targets *HES1* and *HEY* are predominantly expressed in endometrial epithelial cells and are scarce in stromal cells. In addition, JAG1 induces Notch signaling activity in the stromal cells resulting in regulation of 452 genes (Mikhailik et al. 2009). It is important to note that this analysis was performed following cell isolation and culturing. Other investigation has demonstrated that NOTCH1 and NOTCH3 primarily localize to the glandular epithelium, whereas NOTCH4 localize to the stroma (Cobellis et al. 2008; Mitsuhashi et al. 2012).

During menstrual cycle the human endometrium undergoes various phases including proliferative phase of endometrium regeneration, followed by a mid-secretory phase in which endometrial stromal fibroblasts undergo differentiation known as decidualization to secretory "epithelioid" cells and last late-secretory phase where the endometrium is shed in a nonpregnancy cycle. When menopause begins, the endometrium loses the ability to proliferate and differentiate and loses its functional layers (Salamonsen 2019; Mori et al. 2012; Evans et al. 2016). Notch pathway is active in the human endometrium and highly dynamic; expression patterns of Notch receptors and ligands are different according to cell type and menstrual cycle phase (Mikhailik et al. 2009; Mitsuhashi et al. 2012). Notch signaling is more active in cycling endometrium than in menopause, as the expression levels of three Notch signaling molecules, NOTCH1, NOTCH4, and JAG1, are decreased in postmenopause endometrium (Cobellis et al. 2008).

There are only a few reports regarding the role of Notch signaling in remodeling of the endometrium, and the available data is sometimes conflicting (Table 4.1). Mitsuhashi et al. have found no cyclic changes in expression of NOTCH1, JAG1, and DLL4 proteins during menstrual cycle. Only receptor NOTCH3 expression level was increased during the secretory phase in the stroma cells. The expression of these signaling molecules was investigated predominantly in glandular cells of the normal endometrium (Mitsuhashi et al. 2012). Schuring et al. have not detected cycle-dependent changes in expression of NOTCH1 and NUMB (a negative regulator of Notch) when compared to proliferative and secretory endometrium (Schuring et al. 2018). Meanwhile Cobelis et al. have performed immunohistochemical analysis of 60 samples of physiological endometrium (20 of each in proliferative and secretory phase and in menopause) and observed an increased expression level of NOTCH1 and JAG1 in the secretory phase and the opposite trend for NOTCH4. Authors proposed that these results indicate the role of NOTCH4 in cell proliferation as an attribute of proliferative phase of the cycle, whereas NOTCH1 possibly associates with cell differentiation as a characteristic of secretory phase in the human endometrium (Cobellis et al. 2008). These opposite results were obtained despite the fact that Mitsuhashi and Cobelis groups had used the same antibodies against NOTCH1 for immunodetection, however at different concentrations. Suzuki et al. have also shown the decreased level of NOTCH4 expression during secretory phase if

Table 4.1 Changes of Notch signaling molecules in cycling endometrium secretory phase compared to proliferative phase

	Secretory phase
NOTCH1	↕[a, b] ↑[c]
NOTCH2	ND
NOTCH3	↓[e1]↕[e2]
NOTCH4	↓[c, d]
JAG1	↕[a] ↑[c, e1]
JAG2	ND
DLL1	↑[e2]
DLL4	↕[a] ↑[f]

[a]Mitsuhashi et al. (2012); [b]Schuring et al. (2018); [c]Cobellis et al. (2008); [d]Suzuki et al. (2000); [e]Van Sinderen et al. (2014); [f]Cuman et al. (2014)
1, luminal epithelium; 2, glandular epithelium; *ND* not determined

compared to the proliferative phase (Suzuki et al. 2000). The immunostaining of the endometrium has demonstrated that the expression of NOTCH1 receptor increased significantly in both glandular and luminal epithelium in the mid-secretory phase in comparison to the early- and the late-secretory phases. While NOTCH3 increased in the luminal epithelium during proliferative phase compared to secretory phase, no changes in expression were detected in glandular epithelium. Ligand JAG1 was detected upregulated in the luminal epithelium, and ligand DLL1 and NUMB were found in the glandular epithelium in the mid-secretory compared to the proliferative phase (Van Sinderen et al. 2014). It has been determined that DLL4 expression increases in the secretory phase of the menstrual cycle in both glandular and luminal epithelium (Cuman et al. 2014). Previously, it has been shown that NOTCH mediates uterine stromal decidualization by preventing stromal fibroblast apoptosis and regulates gene expression and cytoskeleton reorganization in mouse (Afshar et al. 2012). It has been revealed, by microarray studies, that *NOTCH2*, *NOTCH3*, and *JAG2* are expressed by the trophectoderm (polarized transporting single cell layer) of human blastocysts (Aghajanova et al. 2012).

The endometrium is involved in implantation and placental formation during establishment of pregnancy. Disorders in this process are a major reason for infertility. Critical role in implantation plays interaction of blastocyst and the endometrium. It was also demonstrated that Notch signaling is involved in implantation and placentation (Cuman et al. 2013; Cuman et al. 2014). NOTCH1, JAG1, and DLL1 are downregulated in the endometrium of women with unexplained infertility (Van Sinderen et al. 2014).

Notch Signaling in the Endometriosis

Endometriosis is a gynecological condition characterized by the growth of ectopic endometrial cells outside the uterus. This tissue similarly to eutopic endometrium undergoes regeneration and shedding during the menstrual cycle. Endometriosis affects one in ten women of childbearing age and infertility is often associated with this disease (Hickey et al. 2014). Endometriosis shares mechanisms of estrogen stimulation and chronic inflammation with endometrial cancer; therefore it may be associated with this type of cancer (Yu et al. 2015). The deregulated expression components of the Notch signaling in endometriosis suggest an involvement of this pathway in the pathogenic process. Su et al. reported that Notch receptors NOTCH1 and NOTCH4, ligands JAG2 and DLL4, as well as target genes HEY1and HES5 were downregulated in eutopic endometrium of endometriosis patients suggesting that suppressed Notch signaling contributes to decidualization defects and is responsible for decreased fertility in woman with endometriosis (Su et al. 2015). NOTCH2 has also been shown as a regulator of decidualization (Otti et al. 2014). It was demonstrated that NOTCH1, JAG1, JAG2, and survivin significantly decrease in women with endometriosis, polycystic ovary syndrome, or repeated implantation failure, concluding that Notch signaling molecule might be associated with implantations problems and poor outcomes observed in these diseases (Amjadi et al. 2019). In addition, the expression of gene FOXO1 (NOTCH1 coactivator) was activated in decidualization. Interestingly, NOTCH1 also regulate FOXO1 expression. In the case of endometriosis, the suppression of Notch signaling results in decreased FOXO1 expression and decidualization failure (Brar et al. 2001). In contrast to the aforementioned studies, several groups have obtained opposite results. Expression of NOTCH1 in patients with deep infiltrating endometriosis and NUMB in luminal epithelium was significantly higher as compared with controls (Schuring et al. 2018). In the findings of another group, the expression levels of NOTCH1 and JAG1were upregulated in ectopic endometria than in their eutopic and normal counterparts. Moreover, estrogen regulates cell invasion in the endometriosis via activation of estrogen receptor alpha and the enhancement of Notch signaling (Li et al. 2018). After ultra-deep targeted sequencing, mutations of *NOTCH1* and

NOTCH2 genes were observed in the ectopic endometrium and atypical endometriosis, but not in normal endometrium tissue (Er et al. 2016).

Endometrial Cancer

Endometrial cancer (EC) is the sixth most common malignancy among women and 15th cancer in general. The rates of new diagnoses have been rising by about 1% each year. The frequency of this cancer varies among different countries – the incidence of this disease is highest in Central and Eastern Europe and North America, and it is lowest in Middle and Western Africa. Importantly, the incidence of EC is rising in high-income countries; this might be attributable to high rates of obesity, physical inactivity, late menopause, and extended life expectancy. Endometrial cancer accounts for 2% of cancer deaths in women. Fortunately, EC often causes specific symptoms at early stages, and when diagnosed at an early stage, 5-year survival rate is relatively high, reaching 69%. On the other hand, a delayed diagnosis leads to advanced stage and lower chance of survival. The majority of EC are sporadic, and 5–10% of women inherit cancer susceptibility (Sundar et al. 2017; Amant et al. 2018; Ferlay et al. 2018; World Cancer Research Fund 2019).

Endometrial carcinoma has been traditionally classified into two histological types described by Bokhman (Bokhman 1983). Type I tumors make up 80–90% of endometrial cancers and are typically characterized by a low-grade endometrioid histology (endometrioid endometrial cancer, EEC), arising on a background of atypical hyperplasia. EEC is characterized by estrogen and progesterone receptor positivity and has a favorable prognosis in most cases. Factors leading to an excess of estrogen relative to progesterone are associated with this type of cancer (Sanderson et al. 2017; Amant et al. 2018). Type II cancer is determined in 10–20% of endometrial cancers. This type of cancer is associated with typically high-grade non-endometrioid histology (serous endometrial cancer, SEC; clear cell endometrial cancer, CCEC; uterine carcinosarcoma, UCS), arising in atrophic endometria, and is usually estrogen independent. The precursor of lesion in this type endometrial carcinoma is not yet fully established. This cancer type has higher risk for metastases and less favorable prognosis (Pathiraja et al. 2013; Akhtar et al. 2019).

Aside from morphologic differences between type I and type II, the endometrial cancers are distinguished by genetic alterations. The mutations of the tumor suppressor gene PTEN (phosphatase and tensin homolog) are the most frequent genetic alteration (up to 83%) in endometrioid endometrial cancer. This mutation coexists with other mutations in PIK3CA (phosphatidylinositol 3-OH kinase), CTNNB1 (which encodes β-catenin), KRAS (proto-oncogene, GTPase), and ARID1A (AT-rich interaction domain 1A) and defects in DNA mismatch repair. Mutations in tumor protein TP53, component of ubiquitin ligase Skp1-Cullin1-F-box complex FBXW7 (F-box with 7 tandem WD40), and PPP2R1A (protein phosphatase 2 scaffold subunit alpha) are found in non-endometrioid endometrial cancer. Other frequent alterations in this type of cancer is the overexpression of oncogene HER2/neu (erb-b2 receptor tyrosine kinase 2) and p16 (cyclin-dependent kinase inhibitor 2A, CDKN2A) loss of function leading to uncontrolled cell growth and aneuploidy (McConechy et al. 2012; Matias-Guiu and Prat 2013; O'Hara and Bell 2012).

However, the accuracy of histologic classification is not sufficient in order to distribute patients into optimal treatment subgroups, since various endometrial cancer types may exhibit shared characteristics. Recently, significant progress has been made in understanding molecular events in EC, and a division of tumors into distinct prognostic groups was suggested. The Cancer Genome Atlas (TCGA) provides the most comprehensive molecular study, involving whole-genome sequencing, exome sequencing, MSI assay, and a copy number analysis. Endometrial cancers could be classified into four distinct groups: (1) POLE (polymerase ξ exonuclease) ultramutated, which have *POLE* exonuclease domain mutations; (2) MSI hypermutated tumors, which have MSI-H (microsatellite instability-

high) status and have hypermethylated *MLH1* promoter; (3) copy-number low, MSS (microsatellite stabile) tumors, characterized by a low copy number aberrations and frequent *CTNNB1* mutation; and (4) and copy-number high which are characterized by high-level copy number alterations and frequent *TP53* mutation. Women in POLE-mutated subgroup exhibit the best prognosis, whereas women from copy-number high subgroup have the worst prognosis in progression-free survival. This classification is currently not applied in routine clinical practice, but due to the evolution of the methodologies involved, it will, hopefully, be ready in the near future (Cancer Genome Atlas Research et al. 2013; Urick and Bell 2019; Carlson and McCluggage 2019).

Notch Signaling in the Endometrial Cancer

Expression of Notch Signaling Components Deregulated expression of Notch receptors and Notch ligands has been found in an increasing number of human solid tumors. However, there are only a limited number of reports of Notch signaling in the endometrial cancer. Moreover, as in the case of normal endometrium/endometriosis, the role of Notch signaling in endometrial cancer is ambiguous and seems to depend on analyzed Notch receptor/ligand as well as on the analysis methods. For Notch signaling component expression analysis, in most cases immunohistochemical staining was used, and the expression in the endometrium was compared to normal tissue from separate patients. Suzuki et al. have published the first report about Notch signaling in endometrial cancer in 2000 (Suzuki et al. 2000). They reported that endometrial cancer cells express a significantly lower level of NOTCH4 compared to normal endometria proposing the role of NOTCH4 in endometrial cancer development. Cobellis et al. studied normal (n = 60) and pathological (n = 60) endometrium samples by immunohistochemical analysis. They detected Notch signaling changes in different normal endometrium phases. The elevated expression of NOTCH1 in hyperplasia and carcinoma compared to polyps was found, whereas NOTCH4 and JAG1 expression decrease correlated to histological grade. In support, the expression of NOTCH1 and NOTCH4 correlated to p21 and cyclin D expression level (Cobellis et al. 2008). Mitsuhashi et al. demonstrated, using immunohistochemistry, the expression of NOTCH1, NOTCH3, JAG1, and DLL4 proteins was higher in endometrial cancer (n = 76) versus normal endometrium (n = 37) from non-cancer patients. Additionally, the elevated level of NOTCH1 correlated with cancer aggressiveness such as invasion into the myometrial layer and metastasis. High expression of NOTCH1 and JAG1 was associated with poor patient's prognosis (Mitsuhashi et al. 2012). Mori et al. using immunohistochemistry staining also obtained similar results that NOTCH1 expression in endometrial adenocarcinoma (n = 21) was significantly higher than in normal endometrium (Mori et al. 2012). When the amount of proteins NOTCH1, NOTCH3, NOTCH4, and JAG2 was determined by Western blot analysis of endometrial cancer and adjacent nontumor tissue from the same patient, the level of proteins NOTCH1 and NOTCH3 was unchanged. Meanwhile the relative amount of proteins NOTCH4 and JAG2 was decreased in the majority of stage I endometrial cancer samples, compared to nontumor endometrium of the same patient (Sasnauskiene et al. 2014).

Gene expression analysis between normal and malignant patient samples showed significant elevation of *JAG2* level in endometrial cancer tissues, but it has no impact on cancer patient survival (Townsend et al. 2019). Jonusiene et al. found the decreased expression of Notch receptors (*NOTCH1*, *NOTCH2*, *NOTCH3*, and *NOTCH4*), ligands (*JAG1*, *JAG2*, and *DLL1*), and Notch target gene *HES1* in endometrial cancer tissue compared to normal endometrium of the same patient at mRNA level. For this analysis the samples of endometrial cancer and adjacent nontumor endometrial tissue from the same woman were used (n = 50) (Jonusiene et al. 2013). This study supports the notion that Notch signaling is less active in endometrial cancer and supposes

that it can function as tumor suppressor. In Williams et al. investigation, it was discovered that early endometrial cancer cells lose the apicobasal polarity in the low-grade endometrial cancer. In addition, it was observed the mislocalization of Notch receptor. The decreased expression of *NOTCH4* receptor, ligand *JAG1*, and downstream targets *HES1* and *HEY1* in low-grade endometrial cancer was detected by RT-PCR analysis, indicating that overall Notch signaling is suppressed in low-grade endometrial cancer, while the expression of *NOTCH1* and *NOTCH2* was unchanged (Williams et al. 2017).

Polychronidou et al. analyzed the expression of NOTCH2, NOTCH3, and JAG1 in endometrial carcinoma tissue ($n = 204$) by immunostaining. It was found that more than 70% of tumor were negative for all three proteins. The study has been performed only using cancer tissues. The analysis showed that expressions of NOTCH2 and JAG1 have opposite prognostic impacts. The expression of NOTCH2 has the unfavorable prognostic impact in endometrial cancer, while JAG1 expression reflects a favorable prognosis. Moreover, expression of JAG1 was favorable in the absence NOTCH2/3 and was very similar to patients with low or undetectable expression of all three markers. The expression of NOTCH3 did not yield significant results possibly, due to small number of NOTCH3-positive patients (Polychronidou et al. 2018).

The data of a retrospective study obtained from The Cancer Genome Atlas (545 tumor and 35 adjacent tissues) concluded that expression of Notch ligand DLL3 was upregulated in endometrial cancer tissues. Evaluation depends on patient age, FIGO stage, and grade. It was also discovered that upregulation of DLL3 expression was associated with the shortest overall survival in patients with endometrial cancer (Wang et al. 2018).

All available data concerning Notch signaling member's expression in endometrial cancer are summarized in Table 4.1. All investigations coincide on the only one point: the expression levels of NOTCH4 protein and mRNA are decreased in endometrial cancer. Meanwhile, the expression status of other Notch signaling members is scarce and sometimes contradictory, and more data are needed to draw the conclusions (Table 4.2).

Table 4.2 Expression analysis of Notch signaling members in endometrial cancer

	Protein expression	mRNA expression
NOTCH1	↑[b,c,d] ↕[e]	↓[g] ↕[h]
NOTCH2	ND	↓[g] ↕[h]
NOTCH3	↑[c] ↕[e]	↓[g]
NOTCH4	↓[a,b,e]	↓[g,h]
JAG1	↓[b] ↑[c]	↓[g,h]
JAG2	↓[e]	↓[g] ↑[f]
DLL1	ND	↓[g]
DLL3	ND	↑[i]
DLL4	↑[c]	ND
HES1	ND	↓[g,h]
HEY1	ND	↓[h]

[a]Suzuki et al. (2000); [b]Cobellis et al. (2008); [c]Mitsuhashi et al. (2012); [d]Mori et al. (2012); [e]Sasnauskiene et al. (2014); [f]Townsend et al. (2019); [g]Jonusiene et al. (2013); [h]Williams et al. (2017); [i]Wang et al. (2018); *ND* not determined

Stem Cells in Endometrial Cancer A small population of adult stem cells, including epithelial progenitors, mesenchymal stem cells, and side-population cells, have been identified in the human endometrium. These cells contribute to regenerative capacity of the endometrium (Evans et al. 2016). Cancer stem cells (CSCs) are cells with stem-like properties crucial for generation of neoplastic cell population; they are responsible for invasiveness and formation of drug resistance (Hanahan and Weinberg 2011; Carvalho et al. 2015). Different molecules were studied as markers of CSC in endometrial cancer, including CD133, CD44, CD117(c-kit), and aldehyde dehydrogenase 1 (ALDH1) (Tempest et al. 2018; Giannone et al. 2019). Different signaling pathways regulate stemness in EC, including Notch signaling.

The cell surface marker CD133 is known as prominin; it identifies stem-like cell population. $CD133^+$ cells have exhibited a more aggressive proliferation in vitro and higher resistance to chemotherapeutic drugs cisplatin and paclitaxel (Elbasateeny et al. 2016). Analysis of endometrial cancer Ishikawa cells, separated into two

$CD133^+$and $CD133^-$ subpopulations, demonstrated the increased level of NOTCH1 protein in cancer stem-like cells $CD133^+$. The blockade of the Notch signaling with γ-secretase inhibitor DAPT suppressed CSC proliferation. Moreover, a treatment of Ishikawa cells with DAPT and other therapeutic target, EGFR inhibitor, was more efficient than treating with any compound alone. Authors concluded that Notch signaling seems to be a promising therapeutic target for CSCs (Shang et al. 2018).

Another stem cells marker in EC is RNA-binding protein Musashi-1. Gotte et al. found an increased protein level of Musashi-1 in endometriosis and endometrial cancer. siRNA silencing of Musashi-1 resulted in decreased expression of NOTCH1 protein and its downstream target HES1 in Ishikawa cells. At the functional level, these changes promote reduced cell proliferation and apoptosis induction (Gotte et al. 2011). It was also shown that patients with upregulated Musashi-1 expression have poor survival rate, which may be an independent prognostic factor for endometrial cancer (Ma et al. 2015).

Crosstalk of Notch and Obesity Signals Increasing body mass index is associated with a significant increase in the risk of endometrial cancer (Reeves et al. 2007; Renehan et al. 2008). It has been demonstrated that in comparison with all obesity-related cancers, increasing body mass index is most strongly associated with endometrial cancer incidence and mortality (Schmandt et al. 2011). Although the correlation between obesity and cancer incidence is identified, the molecular mechanisms linking these processes remain the area of intensive studies. Obesity is characterized by excess of adipose tissue, which drives the dysregulation of complex metabolic and endocrine activities (Crean-Tate and Reizes 2018). Leptin is an adipose tissue-secreted hormone, which correlates with the level of adiposity and body mass index in women. Leptin signaling has been shown to induce breast cancer growth and progression (Ando et al. 2014). It was demonstrated that Notch, IL-1, and leptin crosstalk outcome (NILCO) is involved in the induction of breast cancer cell proliferation and migration, where leptin upregulates Notch ligands, receptors, and target genes (Guo and Gonzalez-Perez 2011). In analogy to breast cancer, the group of Gonzalez-Perez hypothesized that NILCO could be a link between obesity and endometrial cancer progression (Daley-Brown et al. 2015). This group has demonstrated that leptin is an inducer of Notch receptors (NOTCH1–4), ligands (JAG1 and DLL4), and downstream effectors (survivin, HEY2) and leptin (OB-R) and IL-1 (IL-1R tI) receptors in endometrial cancer cells (Daley-Brown et al. 2019). The impact of leptin was higher for the poorly differentiated and more aggressive cell lines An3Ca and KLE, resembling type II endometrial cancer. Leptin also upregulated the expression of NOTCH1, NOTCH3, and NOTCH4 receptors in the more differentiated HEC-1A and Ishikawa cells, resembling more differentiated type I endometrial cancer. Moreover, it was demonstrated that leptin induces cell cycle progression and proliferation of endometrial cancer cells. The importance of leptin signaling for endometrial cancer has to be proved using animal models.

Mutations in Notch-Related Genes DNA repair system plays a crucial role in recognition and repairing of insertions or deletions in microsatellites – the repeated sequences of DNA. Abnormal function of a repair system causes the creation of novel microsatellite fragments resulting in microsatellite instability (MSI). Some cancer types, including endometrial cancer, exhibit higher rates of MSI (16.5%). Higher rates of MSI tumor selectively share alterations in genes of common pathways including Notch and Wnt proposing possibilities of pathway-targeted therapies (Trabucco et al. 2019). Mutations of *NOTCH1* and *NOTCH2* genes were identified in endometriosis-associated ovarian cancer, and it may predispose endometriotic lesion to malignant transformation (Er et al. 2016).

miRNAs A class of a small noncoding RNAs, miRNAs, are important for gene regulation, and they are differentially expressed in various malignant tissues. It was identified that 138 miRNAs

are differently expressed in endometrial cancer in comparison to the normal endometrium. Among deregulated miRNAs was miR-34a, regulating members of Notch family *NOTCH1*, *NOTCH2*, *JAG1*, and *DLL*. In addition, *NOTCH1* was regulated by miR-34* and miR-27b* (Jurcevic et al. 2014). Upregulation of miR-34 led to a significant decrease of *NOTCH1* and *DLL1* at mRNA level, while downregulation led to a significant increase in this mRNA (Jurcevic et al. 2016). Devor et al. reported a significant downregulation of miRNA-181c in endometrial cancer. The decrease of miRNA-181c was in part attributed to upregulation of NOTCH2 (Devor et al. 2017).

Conclusion

The aberrant expression of Notch signaling receptors and ligands suggests that this pathway is important for changes in cycling endometrium and in disorders such as endometriosis or endometrial cancer. There are controversial suggestions concerning the role of Notch signaling in the endometrium that are supported by sometimes contradictory results about expression changes of Notch molecules. Therefore, additional functional studies are required to reveal the importance of Notch signaling for endometrial cancer progression. Future challenges in the field include choosing of the right methods and approaches to analyze the importance of Notch signaling for endometrial cancer. It is necessary to understand how this pathway interacts with other signaling pathways, including Wnt and Hedgehog. These new studies may offer new potential markers for endometrial cancers molecular classification and prognostic or therapeutic targets.

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NOTCH and Esophageal Squamous Cell Carcinoma

5

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Abstract

Esophageal squamous cell carcinoma (ESCC) is a deadly disease that requires extensive research on its mechanisms, prevention, and therapy. Recent studies have shown that *NOTCH* mutations are commonly seen in human ESCC. This chapter summarizes our current understanding of the NOTCH pathway in normal esophagus and in ESCC. In normal esophagus, NOTCH pathway regulates the development of esophageal squamous epithelium, in particular, squamous differentiation. Exposure to extrinsic and intrinsic factors, such as gastroesophageal reflux, alcohol drinking, and inflammation, downregulates the NOTCH pathway and thus inhibits squamous differentiation of esophageal squamous epithelial cells. In ESCC, NOTCH plays a dual role as both a tumor suppressor pathway and an oncogenic pathway. In summary, further studies are warranted to develop NOTCH activators for the prevention of ESCC and NOTCH inhibitors for targeted therapy of a subset of ESCC with activated NOTCH pathway.

Keywords

NOTCH · Esophagus · Esophageal squamous cell carcinoma

Abbreviations

4NQO	4-nitroquinoline 1-oxide
BE	Barrett's esophagus
ESCC	esophageal squamous cell carcinoma
HNSCC	head and neck squamous cell carcinoma
SCC	squamous cell carcinoma

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Introduction

Esophageal cancer affected 17,290 adults and caused 15,850 deaths in the USA in 2018 (Cancer Facts & Figs. 2018). In the world, it is the seventh most prevalent cancer and the sixth leading cause of cancer-related death, with more than 572,000 new cases and 508,000 deaths each year (Bray et al. 2018; Siegel et al. 2017). Two main histological types of esophageal cancer exist, squamous cell carcinoma (SCC) and adenocarcinoma. Human esophageal squamous cell carcinoma (ESCC) develops from precancerous lesions, and its histopathology follows a stepwise pattern of hyperplasia, dysplasia, and SCC. The 5-year survival rate for ESCC is ~18%, a number that reflects late diagnosis, the aggressiveness of the disease, and a lack of effective treatment strategies (Kang et al. 2015; Liu et al. 2016a). Thus, there is a great need to further elucidate the molecular mechanisms and develop better preventive and therapeutic strategies for ESCC.

Recent technological advances in NextGen sequencing enabled the detection of gene mutations in human ESCC samples (Agrawal et al. 2012; Gao et al. 2014; Lin et al. 2014; Liu et al. 2016b, 2017; Qin et al. 2016; Sawada et al. 2016; Shibata et al. 2011; Song et al. 2014; Zhang et al. 2015). Common genomic alterations included single nucleotide variants, copy number alterations, and alterations in multiple signaling pathways. The overall mutation pattern appeared similar to that of head and neck SCC (HNSCC) but distinct from that of esophageal adenocarcinoma and lung SCC. Genes on the NOTCH pathway were frequently mutated in human ESCC (Kang et al. 2015; Liu et al. 2016a). This observation brings about interesting questions regarding the functional role of the NOTCH pathway in the development of ESCC.

NOTCH in Normal Esophagus

NOTCH pathway is mediated through ligands (JAG1, JAG2, DLL1, DLL3, DLL4) binding to NOTCH receptors (NOTCH1, 2, 3, 4). These receptors are then cleaved to allow its intracellular domain (e.g., NICD1) to be released from the membrane and enter the nucleus to form a transcriptional complex with RBPJ. NICD1 displaces the repressive cofactors bound to RBPJ and recruits a transcriptional activator complex, which initiates transcription of NOTCH downstream effectors like HES1 (Borggrefe and Oswald 2009). In the normal esophagus of rodents and humans, NOTCH1, NOTCH2, and NOTCH3 are highly expressed, whereas NOTCH4 is expressed at a minimal level (Sander and Powell 2004; Zhang et al. 2018). NOTCH3 expression was found to be subject to transcriptional regulation by NOTCH1, and loss of NOTCH signaling in mouse esophagus resulted in NOTCH3 silence (Ohashi et al. 2010). Nevertheless, NOTCH pathway is largely dispensable for maintaining the integrity of squamous epithelium. Although NOTCH1 is the major regulator of squamous differentiation among four NOTCH receptors, even in triple knockout mice (*Notch1, Notch2, Notch3*), the epidermis still formed almost normally except for the phenotypes of squamous hyperplasia and deficient barrier function (Demehri et al. 2009).

Regulation of Squamous Differentiation by NOTCH NOTCH regulates squamous differentiation in the skin (Okuyama et al. 2008) and the esophagus (Ohashi et al. 2010; Ohashi et al. 2011), particularly in the commitment of keratinocytes to terminal differentiation by a HES1-dependent mechanism (Blanpain et al. 2006; Wang et al. 2008). Not surprisingly, NOTCH interacts with key regulators of squamous differentiation, such as P63 (Tadeu and Horsley 2013), IRF6 (Restivo et al. 2011), NRF2 (Wakabayashi et al. 2013; Wakabayashi et al. 2010), and HPV8 E6 (Meyers et al. 2013). Using gene microarray data of mouse esophagus at various developmental stages and ages, we found the NOTCH pathway participates in the development of mouse esophageal epithelium (Chen et al. 2012). Further studies have shown that NOTCH is required for the specification of esophageal progenitor cells from human progenitor stem cells. In agreement with these observations, genetic deficiency of NOTCH components (*Rbpj, Jag1, Jag2*) inhib-

ited squamous differentiation in mouse esophagus (Zhang et al. 2018).

NOTCH **Mutations in Normal Esophagus** It is surprising that *NOTCH1, NOTCH2, and NOTCH3* mutations occurred much more often in physiologically normal human esophageal epithelia (66.2% samples) than in ESCC (15% samples), and distribution pattern of the mutation sites was similar in normal and ESCC samples. Human subjects with ESCC risk factors (alcohol drinking, tobacco smoking, aging) were more likely to carry *NOTCH* mutations than those without these risk factors (Yokoyama et al. 2019; Martincorena et al. 2018; Yizhak et al. 2019). However, few of the mutations were present in all the cells of the normal clones, and many of the *NOTCH* mutations were found in spatially distinct subclones. These data suggest that *NOTCH* mutations are not sufficient to drive carcinogenesis, and some other mutations are needed. *NOTCH* mutations can be either driver or passenger mutations in human ESCC (Chanock 2018; Ciccarelli 2019).

Why normal esophageal epithelial cells are susceptible to *NOTCH* mutations? A recent report based solely on cancer genome sequencing and epidemiological data estimated that DNA replication errors may contribute to 38.9% gene mutations in ESCC, while hereditary mutations and environmental factors contribute to 0.5% and 60.6% mutations, respectively (Tomasetti et al. 2017a). This was believed to be due to a relatively high rate of stem cell division in the esophagus although debates still remain (Wu et al. 2016; Tomasetti et al. 2017b). Interestingly, there was a significant decrease in the rate of stem cell division in the human esophagus with age. In contrast, only a small decrease was observed in the mouse esophagus. These data provide a plausible explanation for the enigmatic age-dependent deceleration in cancer incidence in very old humans but not in mice (Tomasetti et al. 2019).

Response of the NOTCH Pathway to Gastroesophageal Reflux Regurgitation of gastric and duodenal contents (e.g., acid and bile acids) into the esophagus causes heartburn and leads to substantial impairment of quality of life and work productivity. Some subjects with gastroesophageal reflux are further complicated with Barrett's esophagus (BE) when histologic evidence of intestinal metaplasia is present in the human esophageal epithelium. Acid and bile acids inhibited the NOTCH pathway in esophageal squamous epithelial cells (Wang et al. 2018; Yuan et al. 2017; Morrow et al. 2009), supporting an essential role of the NOTCH pathway in squamous differentiation. Moreover, inhibition of the NOTCH pathway favored goblet cell differentiation, which is diagnostic of human BE. Treatment of a rat model of reflux-induced BE with a γ-secretase inhibitor converted the proliferative Barrett's epithelial cells into terminally differentiated goblet cells, whereas the squamous epithelium remained intact (Menke et al. 2010). When human esophageal squamous epithelial cells were stably transfected with an intestinal transcription factor, *CDX2*, these cells formed crypt-like structures, overexpressed differentiation markers of intestinal columnar epithelial cells and goblet cells, and downregulated *NOTCH* pathway genes (Liu et al. 2007). These data support NOTCH inhibition as one of the molecular mechanisms of human BE as a result of exposure to gastroesophageal reflux (Chen et al. 2011).

Response of the NOTCH Pathway to Alcohol Drinking NOTCH pathway was inhibited by ethanol in the pancreas and smooth muscle cells (Schneider et al. 2012; Morrow et al. 2010). Mechanistically, ethanol suppressed the NOTCH pathway through inhibition of γ-secretase proteolytic activity (Hatch et al. 2015). In the esophagus, we first found out that ethanol exposure downregulated PAX9 expression in human esophageal epithelial cells in vitro and mouse forestomach and tongue in vivo *(*Xiong et al. 2018*)*. More importantly, PAX9 was found to be a downstream effector of the NOTCH pathway in esophageal squamous epithelial cells, and ethanol exposure inhibited the NOTCH pathway as well (unpublished data). Consistent with the function of NOTCH in squamous differentiation, we also showed that *Pax9* deficiency in mouse esophagus promoted cell proliferation and

delayed cell differentiation, and PAX9 was downregulated in human ESCC (Xiong et al. 2018).

Response of the NOTCH Pathway to Inflammation Inflammatory cytokines (IL4, IL5, IL1, TNFα) suppressed NOTCH-dependent transcription, NOTCH ligands, and NOTCH1 target genes in human esophageal epithelial cells. These changes contributed to the development of eosinophilic esophagitis and possibly inflammation-associated ESCC (Kasagi et al. 2018).

NOTCH in ESCC

***NOTCH* Mutations in ESCC** Based on the original data from 2 studies of 227 cases of human ESCC (Lin et al. 2014; Song et al. 2014), *NOTCH1*, *NOTCH2*, and *NOTCH3* mutations took place in 8%, 3%, and 1.9% of human ESCC, respectively. Point mutations tended to cluster in the EGF-like repeats and thus potentially resulted in the loss of function (Fig. 5.1a–c). These mutations tended to be mutually exclusive (Fig. 5.1d). *RBPJ* (a key repressor of canonical NOTCH pathway) and *FBXW7* (the substrate-recognition subunit of an SCF-type ubiquitin ligase complex targeting NOTCH1) were also frequently mutated (Chang et al. 2017; Cheng et al. 2016). It was interesting that *NOTCH1* mutation was mutually exclusive with *PIK3CA* mutation. *NOTCH1* mutation was associated with well-differentiated, early-stage malignancy and less metastasis to regional lymph nodes. Patients with *NOTCH* mutations tended to have a worse prognosis than those without (Fig. 5.2). In contrast, patients with *PIK3CA* mutations had better response to chemotherapy and longer survival time than those without (Song et al. 2016). Moreover, lower expression of NOTCH1 was associated with poorer prognosis than higher expression after adjustment for age, sex, tumor stage, smoking, and alcohol consumption (Qin et al. 2016).

NOTCH as a Tumor Suppressor Pathway In the normal esophagus, NOTCH functions as a tumor suppressor (Nowell and Radtke 2017). Exposure to an oro-esophageal carcinogen, 4-nitroquinoline 1-oxide (4NQO), caused loss of NOTCH1 expression in the basal cells of normal esophageal squamous epithelium, as well as *Notch1* mutations. Loss of *Notch1* in the squamous epithelial cells caused spontaneous SCC in the skin, but not the esophagus. However, loss of *Notch1* promoted 4NQO-induced oro-esophageal SCC (Nyman et al. 2018; Sawangarun et al. 2018). Similarly, NOTCH inhibition in mouse esophagus increased the number and size of tumors following exposure to an esophageal carcinogen, diethylnitrosamine (Alcolea et al. 2014).

Using the lineage tracing technique in mice carrying a conditional dominant-negative mutant of *Maml1* (a transcriptional coactivator for NOTCH), Alcolea et al. found that NOTCH inhibition prevented differentiation of mutant progenitor cells and promoted differentiation of neighboring wild-type progenitor cells in mouse esophagus (Alcolea et al. 2014). Such combined effects led to clonal expansion with mutant cells eventually replacing the entire epithelium, supporting the idea that NOTCH mutation promotes field change in the human esophageal epithelium (Yokoyama et al. 2019; Martincorena et al. 2018; Yizhak et al. 2019).

NOTCH as an Oncogenic Pathway However, the NOTCH pathway plays a dual role in carcinogenesis, both oncogenic and tumor suppressor, depending on the cellular and genetic context (Nowell and Radtke 2017; Lobry et al. 2011; Ranganathan et al. 2011; Zhong et al. 2015; Sun et al. 2014). It is believed that NOTCH pathway turns to be oncogenic during the process of carcinogenesis. In ESCC, cellular senescence checkpoint functions (e.g., P16-Rb, P14, P53) determined differential NOTCH1-dependent oncogenic and tumor suppressor activities (Kagawa et al. 2015). Activated NOTCH1 was detected in a small subset of cancer cells at the invasive front in human ESCC, which correlated with higher tumor aggressiveness. NOTCH1

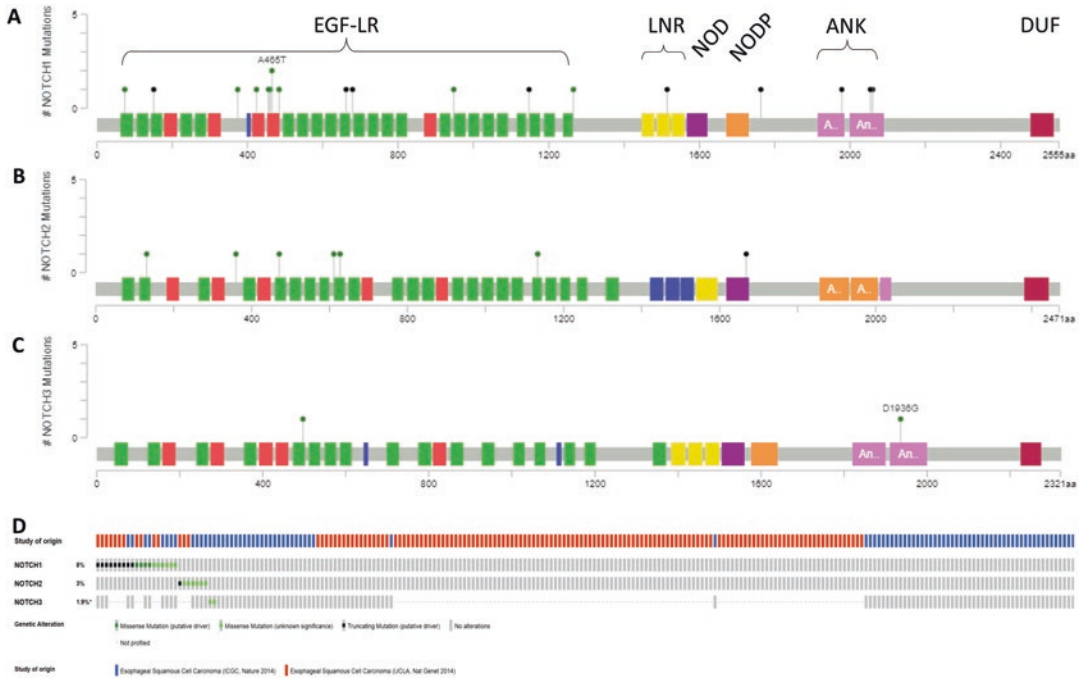


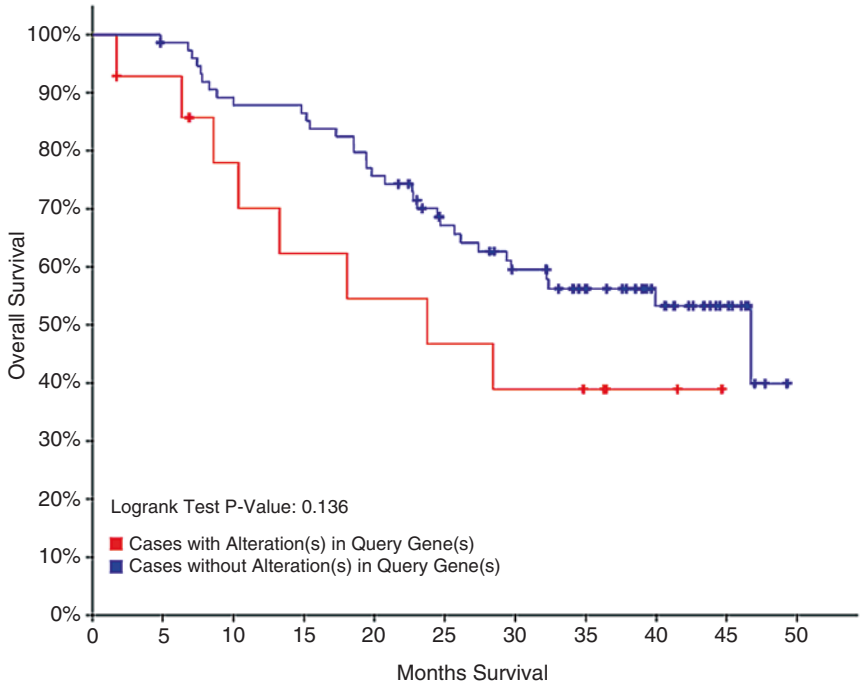
Fig. 5.1 *NOTCH* mutations in human ESCC. These plots are downloaded from the TCGA database (www.cbioportal.org) based on the original data from 2 studies of 227 cases of human ESCC (Lin et al. 2014; Song et al. 2014). EGF-LR, epidermal growth factor-like repeat; LNR, LIN12-NOTCH repeat; NOD and NODP, NOTCH protein domain; ANK, ankyrin repeats; DUF, domain of unknown function

facilitated not only epithelial-mesenchymal transition but also TGFβ-mediated tumor initiation by increasing the number of cancer stem cells (Natsuizaka et al. 2017).

Similar to ESCC, NOTCH activity is contextual, and NOTCH in HNSCC is considered to have a dual role as a tumor suppressor and an oncogene (Fukusumi and Califano 2018), at least in a subset of HNSCC based on genomics data (Sun et al. 2014). NOTCH4-HEY1 pathway induced epithelial-mesenchymal transition in cultured cells (Fukusumi et al. 2018). Both deficiency and activation of *Notch1* promoted oral squamous cell carcinogenesis in a genetic model driven by HPV E6/E7 and $Kras^{G12D}$ (Zhong et al. 2015). Inactivation of the NOTCH pathway by a dominant-negative form of *Maml1* promoted HNSCC induced by 4NQO, especially in the presence of *p53* mutation or HPV16 E6/E7 oncoproteins (Nyman et al. 2018). On the other hand, RBPJ acted as a tumor-promoting function in HNSCC (Al Labban et al. 2018).

Conclusion

Considering its function as a tumor suppressor, NOTCH activators may be used for the prevention of ESCC. Chemical NOTCH activators, e.g., resveratrol, valproic acid, chrysin, hesperetin, thiocoraline, and N-methylhemeanthidine chloride (Wyche et al. 2014; Patel et al. 2014; Yu et al. 2013; Pinchot et al. 2011; Greenblatt et al. 2007; Ye et al. 2016), may be further tested for their protective effect on ESCC. Other than chemical NOTCH activator, a NOTCH3 antibody is also known to activate NOTCH (Li et al. 2008). NOTCH activation may also be achieved through inhibition of negative regulators of the NOTCH pathway, such as FBXW7 or NUMB. On the other hand, NOTCH inhibition needs to be



	No. of Cases (Total)	No. of Cases (Deceased)	Median Survival (Months)
Cases with mutation	14	8	23.75
Cases w/o mutation	74	33	46.72

Fig. 5.2 *NOTCH* mutations and prognosis of human ESCC. The plot is downloaded from the TCGA database (www.cbioportal.org) based on the survival data from 1 study of 88 cases of human ESCC (Song et al. 2014)

explored as a potential therapy for a subset of ESCC with activated NOTCH pathway.

It should be noted that the NOTCH pathway is complicated in the esophagus just like in many other organs. NOTCH pathway in the normal esophagus behaves differently from that in ESCC. NOTCH target genes in the normal esophagus and those in ESCC need to be identified for functional characterization of the NOTCH pathway in these contexts. Moreover, it remains to be elucidated how NOTCH pathway discriminates between distinct ligands as well as receptors in these contexts (Nandagopal et al. 2018). If we further consider NOTCH pathway in the tumor microenvironment (Meurette and Mehlen 2018) and noncanonical NOTCH pathway (Steinbuck and Winandy 2018), it is obvious that a lot more studies are warranted to elucidate the sophisticated role of the NOTCH pathway in ESCC.

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Notch Signaling and Liver Cancer

6

Kazunori Kawaguchi and Shuichi Kaneko

Abstract

Interactions between liver cells are closely regulated by Notch signaling. Notch signaling has been reported clinically related to bile duct hypogenesis in Alagille syndrome, which is caused by mutations in the Jagged1 gene. Notch activation and hepatocarcinogenesis are closely associated since cancer signaling is affected by the development of liver cells and cancer stem cells. Gene expression and genomic analysis using a microarray revealed that abnormalities in Notch-related genes were associated with the aggressiveness of liver cancer. This pattern was also accompanied with α-fetoprotein- and EpCAM-expressing phenotypes in vitro, in vivo, and in clinical tissues. Hepatitis B or C virus chronic infection or alcohol- or steatosis-related liver fibrosis induces liver cancer. Previous reports demonstrated that HBx, a hepatitis B virus protein, was associated with Jagged1 expression. We found that the Jagged1 and Notch1 signaling pathways were closely associated with the transcription of covalently closed circular hepatitis B virus DNA, which regulated cAMP response element-binding protein, thereby affecting Notch1 regulation by the E3 ubiquitin ligase ITCH. This viral pathogenesis in hepatocytes induces liver cancer. In conclusion, Notch signaling exerts various actions and is a clinical signature associated with hepatocarcinogenesis and liver context-related developmental function.

Keywords:

Notch signaling · Liver cancer · Hepatocellular carcinoma · Cholangiocarcinoma · Liver cancer stem cells · α-Fetoprotein · Hepatitis B virus · Covalently closed circular DNA

Notch Signaling and the Liver

Notch signaling is closely associated with liver regeneration because tissue context is more complex in the liver than in other organs. Hepatocytes develop from hepatoblasts, which are derived from the endoderm, as are bile duct cells. Endothelial and sinusoidal cells are derived from the mesoderm, while stellated cells, which are located between the hepatic fossa and horizontal septum, are also derived from the mesoderm. Notch signaling is a cell-to-cell contact signaling

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system that plays an important role in the liver due to the tissue context-dependent interactions between cells derived from different sources. Abnormalities in Notch signaling in the liver can lead to the development of fatal disorders.

Tissue context-related research has been developed in recent years using organoid models, and the generation of a hepatobiliary organoid has been reported (Vyas et al. 2018). A hepatobiliary-pancreatic organoid was also developed recently (Koike et al. 2019). The formation of bile ducts in this organoid was regulated by the gene encoding Hes family bHLH transcription factor 1 (HES1), which is located downstream of the Notch receptor. This phenomenon was dependent on the interactions of different types of neighboring cells via Notch signaling.

The relationship between Notch signaling and development has been reported previously and is conserved across animal species (Adams and Jafar-Nejad 2019). Notch signaling is associated with liver homeostasis, and this modulation results in decreased fenestration and increased basement membrane in liver sinusoidal endothelial cells (Duan et al. 2018; Geisler and Strazzabosco 2015). Moreover, endothelial Notch activation leads to the dedifferentiation of liver sinusoidal endothelial cells and the acceleration of liver fibrogenesis through endothelial nitric oxide synthase-soluble guanylate cyclase signaling and alters the angiocrine profile of liver sinusoidal endothelial cells to compromise hepatocyte proliferation and liver regeneration. A study using zebrafish examining the effects of epigenetic factors on biliary epithelial cell-derived hepatocyte progenitor cells (HPCs), which can mediate liver regeneration, reported that a Notch3 mutation led to a severe loss of hepatocytes, which was associated with mainly Sox9b and Cdk8 (Ko et al. 2019). Notch signaling is closely associated with liver fibrosis. Activated hepatic stellate cells, which are a component of the HPC niche, express Jagged1, which plays a central role in the differentiation of HPCs into biliary epithelial cells, and antifibrosis treatment results in HPC-mediated liver regeneration (Kitade et al. 2019).

Close Relationship Between Notch Signaling and Liver Diseases

Notch signaling has a central role in the formation of different cell types in a context-dependent manner, which underlies the organization of tissues and organs. Abnormalities in Notch signaling are related to diseases that affect the regeneration of specific organs. Alagille syndrome is a congenital disorder of the liver and is related to Notch signaling via mutations in the Jagged1 gene (Oda et al. 1997; McDaniell et al. 2006). However, Notch signaling is also implicated in other liver diseases. Studies of these diseases have enabled the characterization of Notch ligands and receptors, showing that each molecule has a different function, thereby leading to different types of liver pathogenesis (Table 6.1).

Notch1 is associated with the tumorigenicity of hepatocytes, and biliary differentiation from HPCs is controlled by autophagy via the Notch1 signaling pathway (Zeng et al. 2016; Wang et al. 2009). M1 macrophages have a role in alcoholic liver injury via Notch1 signaling (Xu et al. 2015). Notch2 is associated with developmental retardation and bile duct development, since defects in Notch2 result in cholangiocyte hypogenesis (Geisler et al. 2008). Notch3 drives the differentiation and progression of cholangiocarcinoma (Guest et al. 2016). However, the relationship between its activation levels and liver cancer has not been defined, although it is reported to have a role in liver fibrogenesis (Zheng et al. 2013). Notch3 also induces the commitment of HPCs to the hepatocyte lineage (Ortica et al. 2014). Notch4 is associated with a reversible arteriovenous malformation, and a deficiency in Notch4 leads to angiogenesis, vascular remodeling, and the generation of hepatocyte lineage HPCs, resulting in regeneration or tumorigenesis (Lu et al. 2016; Carlson et al. 2005; Ahn et al. 2013).

The upregulation of Notch1, Notch3, and Notch4 expression is associated with liver cancer involving hepatitis B virus (HBV)-X protein (HBx) (Yang et al. 2017; Carlson et al. 2005). As a Notch ligand, Jagged1 mutations or defects are pathogenic for Alagille syndrome, while Jagged2 is not considered to have an important role in

Table 6.1 Roles of Notch ligands and receptors associated with liver pathogenesis and liver cancer

Notch molecules	Related functions with liver pathogenesis	Reference	Association with liver cancer	Reference
Jagged1	Mutation: Alagille syndrome Upregulation: HBV cccDNA transcription	Oda et al. (1997) Wang et al. (2019)	Upregulation: HBx and hepatocarcinogenesis Genomic amplification and upregulation: α-fetoprotein-related poor prognosis	Gao et al. (2007) Kawaguchi et al. (2016)
Jagged2	NR		NR	
DLL1	NR		NR	
DLL3	NR		NR	
DLL4	Upregulation: liver fibrosis and fat deposition, M1 macrophage activation	Fukuda et al. (2012)	Upregulation: HBx and hepatocarcinogenesis	Kongkavitoon et al. (2016)
Notch1	Downregulation: biliary undifferentiation from HPCs by autophagy Upregulation: HBV cccDNA transcription Upregulation: M1 macrophage activation in alcoholic liver injury	Zeng et al. (2016) Wang et al. (2019) Xu et al. (2015)	Upregulation: correlation with β-catenin Upregulation: HBx and hepatocarcinogenesis Upregulation: cancer, poor prognosis	Wang et al. (2009) Kongkavitoon et al. (2016) Ahn et al. (2013)
Notch2	Defect: hypogenesis of cholangiocyte Mutation: Alagille syndrome Upregulation: proliferation of hepatoblasts	Geisler et al. (2008) McDaniell et al. (2006) Ortica et al. (2014)	Upregulation: aggressive behavior and immature morphology of liver cancer cells Upregulation: HPCs: dedifferentiated liver cancer Upregulation: relationship with hepatoblastoma	Hayashi et al. (2015) Dill et al. (2013) Jeliazkova et al. (2013) Litten et al. (2011)
Notch3	Upregulation: hepatocytic-lineage commitment of HPCs Upregulation: liver fibrogenesis	Ortica et al. (2014) Zheng et al. (2013)	Upregulation: differentiation and progression of cholangiocarcinoma	Guest et al. (2016)
Notch4	Upregulation: proliferation of hepatoblasts	Ortica et al. (2014)	Upregulation: cancer, poor prognosis	Ahn et al. (2013)

NR not reported, *HPCs* hepatocyte progenitor cells, *NASH* nonalcoholic steatohepatitis

hepatocytes or liver tissue. Although defects in delta-like 1 (DLL1) stimulate neuronal differentiation and severe somite patterning abnormalities, reports on their relationship to the liver are scarce. Moreover, while DLL3 defects produce abnormalities in somitogenesis and autosomal recessive spondylocostal dysostosis, they show little association with liver diseases (Penton et al. 2012). DLL4 defects result in arteriovenous shunting and severe vascular remodeling defects, and upregulated DLL4 expression has been reported in relation to liver diseases such as liver cancer, nonalcoholic steatohepatitis, and HBx-related tumorigenesis (Kongkavitoon et al. 2016; Iwamoto et al. 2015; Fukuda and Aikawa 2013; Fukuda et al. 2012). These studies indicated that Notch-related ligands and receptors have important roles in organ and tissue formation, especially in vascular tissue, bile ducts, and neurons.

In patients with chronic HBV infection, the repression of Notch receptors is suggested to repress immune regulation, which results in the inhibition of differentiation and proliferation of effector cells, consequently leading to further pathogenesis (Wei et al. 2016). Furthermore, fibrosis-related Notch signaling abnormalities in liver diseases are important for liver cancer research because many liver cancers have a background of progressive fibrosis and inflammation, and these microenvironments are closely associated with tumorigenesis and aggressive carcinogenic characteristics. The DLL-Notch pathway is

associated with liver fibrosis (Nakano et al. 2016; Zheng et al. 2016; Bansal et al. 2015).

Close Relationship Between Notch Signaling, Liver Cancer Pathogenesis, and Liver Cancer Stem Cells

Notch activation and liver carcinogenesis are closely related because cancer signaling is affected by the development of liver cells and the interactions between liver component cells, including bile duct cells, endothelial cells, stellate cells, fibroblasts, and immune cells (Lee et al. 2016). Notch activation can trigger epithelial-mesenchymal transformation, promoting the self-renewal of cancer stem cell-like niches in primary tumors (Liu et al. 2018).

Some molecules that are affected by Notch signaling have been reported. The pro-oncogenic function of mastermind2 targets genetic alterations in various types of cancer, and it is associated with Notch activation even in hepatobiliary neoplasms (Kochert et al. 2011; Nemoto et al. 2007; Lee et al. 2006). Genetic analysis has shown that mice overexpressing Notch intracellular domain (NICD), which is the active form of Notch1, present with a cluster of liver cancers (Villanueva et al. 2012). Notch2 overexpression causes HPCs to spontaneously develop into dedifferentiated liver cancer cells (Jeliazkova et al. 2013), and Notch-induced malignant hepatocyte transformation is associated with the downregulation of hepatocyte-associated genes, including Sox9 (Liu et al. 2016). Liver cancer has been reported frequently, even among other hepatomas except for hepatocellular carcinoma (HCC). Notch2 is associated with the aggressiveness of HCC and hepatoblastoma (Hayashi et al. 2015; Falix et al. 2014; Dill et al. 2013; Litten et al. 2011). RUNX3 is reported to have a role in the suppression of liver cancer and Jagged1 upregulation (Gao et al. 2010; Nishina et al. 2011). Jagged1/Notch1 signaling is associated with upstream YAP and Hippo signaling and β-catenin signaling (Wang et al. 2009; Kim et al. 2017). Moreover, gamma secretase inhibitors (GSIs) are effective angiogenic factors for liver cancer (Yimlamai et al. 2014; Tschaharganeh et al. 2013; Kim et al. 2017).

In the development of cholangiocarcinoma, NICD associated with protein kinase B signaling stimulates the malignant differentiation of hepatocytes (Zhu et al. 2014). Notch contributes to the formation of intrahepatic cholangiocarcinoma (ICC) arising from the conversion of hepatocytes rather than cholangiocytes activated by Notch signaling (Sekiya and Suzuki 2012). This indicates that Notch signaling activates the malignant characteristics of hepatomas and ICC.

Liver tumorigenesis caused by HBV is associated with HBx directly or with disease progression (Yang et al. 2017; Trehanpati et al. 2012; Gao et al. 2016; Wang et al. 2010; Gao et al. 2007), and the hepatitis C virus core protein is regulated by gamma secretase, which also regulates Notch signaling (Weihofen et al. 2002; Weihofen et al. 2003).

Some liver cancers are associated with angiogenesis, which can be regulated by GSIs, to reduce the development of hepatoma cells, especially α-fetoprotein (AFP)-upregulated cells (Iwamoto et al. 2015). Blocking of Notch signaling molecules, such as with short hairpin RNA (shRNA) targeting Jagged1, results in an effective decrease of AFP-upregulated hepatoma cells (Kawaguchi et al. 2016). Thus, gamma secretase may be a target for liver cancer therapy, although the phenotype might be restricted.

Notch signaling is involved in carcinogenesis of the liver from the aspect of cancer stem cells (Lu et al. 2016). The Notch signaling pathway is a crucial determinant of cell fate during development and disease in several organs. In liver cancers, Notch signaling is involved in biliary tree development and tubulogenesis and also has a significant role in the development of HCC and ICC. CD90 is a marker of liver cancer stem cells (LCSCs), and high CD90 expression is reported to correlate with Notch signaling (Luo et al. 2016). Notch signaling is activated in $CD90^+$ cells, and inhibition of Notch signaling in $CD90^+$ LCSCs decreases tumorigenicity, cell invasion, migration, and the expression of stem cell-related genes. Moreover, cancer stem cell features are

facilitated by stimulating G1-S cell cycle transition and inhibiting Notch signaling-mediated apoptosis.

Notch signaling is also important for LCSCs together with Wnt/β-catenin signaling (Wang et al. 2016). Using NOD/SCID mouse models, Notch and Wnt/β-catenin signaling were shown to play important roles in increasing the stemness of LCSCs. The expression of the active form of Notch1 (i.e., NICD) depends on Wnt/β-catenin pathway activation. Moreover, Notch1 negatively contributes to Wnt/β-catenin signaling modulation.

The chromatin modification factor lysine-specific demethylase 1 (LSD1) is highly expressed in LCSCs in HCC, where its expression is decreased during differentiation (Liu et al. 2018). Notch signaling activates LSD1 through the induction of sirtuin 1, leading to the deacetylation and activation of LSD1 and self-renewal of LCSCs. LSD1 and Notch3 expression is associated with poor patient survival. Other reports demonstrated that inducible nitric oxide synthase promotes the development of $CD24^{+}CD133^{+}$ LCSCs and is dependent on Notch1 activation (Wang et al. 2018), which in turn is associated with TACE/ADAM17 activation. Since Notch is cleaved by ADAM family proteins, the progression of LCSCs is closely related to Notch activation and poor survival.

Organoid-related research is also an important source of information regarding Notch signaling abnormalities, and liver cancer-initiated organoids can be generated from directly reprogrammed human hepatocytes (Sun et al. 2019). Liver cancer can be induced in this model by inactivating p53 and RB proteins. Using this model, it was demonstrated that the RAS-induced lineage conversion of hepatocytes to intrahepatic cholangiocarcinoma cells can be prevented by the combined inhibition of Notch and JAK-STAT.

Jagged1 Gene Abnormalities and Liver Cancer

Liver cancer tissue undergoes various genomic changes, and as a Notch-related genomic abnormality, protein O-glucosyltransferase 1 copy number variation (CNV) is associated with liver cancer (Annani-Akollor et al. 2014; Thakurdas et al. 2016). Moreover, we found that the copy number of Jagged1 was increased in liver cancer tissue (Kawaguchi et al. 2016). We analyzed clinical samples and showed that Jagged1 genomic amplification and overexpression in AFP-producing cells were associated with liver cancer, the malignant phenotype, and poor overall survival.

Specific genes for CNV in liver cancer cells were investigated using microarray-based comparative genomic hybridization (CGH) to determine whether these factors were related to clinical outcome. Chromosome 20p, which includes Jagged1, was found to be amplified in several types of hepatoma cells, and its mRNA was upregulated according to AFP expression levels.

We found that Notch signaling inhibition using Jagged1 shRNA and GSIs significantly suppressed the growth of AFP-expressing cells with the suppression of downstream genes. In detail, two GSIs (L-685,458 and N-[N-(3,5-difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester (DAPT)) were used to inhibit the Notch signaling pathway in different hepatoma cell lines. Notch signaling was inhibited in Huh7 and HepG2 cells, which are AFP-expressing cell lines, whereas there was no significant inhibition in the non-AFP-expressing HLE and SKHep1 cell lines (Fig. 6.1a).

We assessed the effectiveness of GSIs in epithelial cell adhesion molecule-positive ($EpCAM^{+}$) cancer stem cells since Notch signaling plays a role in the functions of stem cells and found that $EpCAM^{+}$ cells were strongly associated with cancer stem cells in hepatomas.

The antitumor effect of Notch inhibitors in mouse hepatoma models was assessed. Slower tumor formation was observed after the administration of L-685,458 and DAPT. Examination of the dynamic tumor status of Huh7-implanted NOD-SCID mice treated with GSIs for an extended period of time revealed tumor necrosis and apoptosis (Fig. 6.1b). We found that EpCAM and HES1 staining was weak in the GSI-treated groups except in necrotic areas, suggesting that

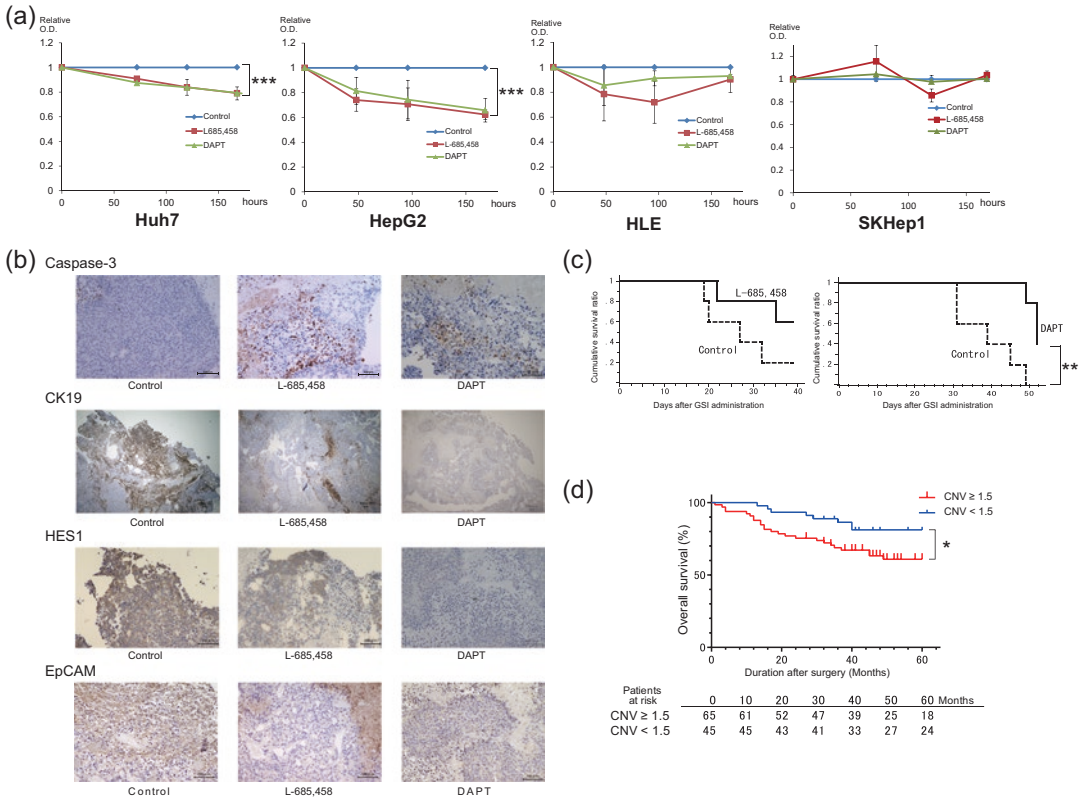

Fig. 6.1 (**a**) Impact of Notch inhibition on AFP-producing cells in vitro. Relative cellular growth curve of hepatoma cell lines after the administration of the γ-secretase inhibitors L-685,458 and N-[N-(3,5-difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester (DAPT). We administered 10 μM of each reagent dissolved in DMSO at 0, 72, 120, and 168 h and blank reagents that included only DMSO (control). ***$P < 0.001$; control versus L-685,458 and DAPT. (**b**) Immunostaining using anti-CK19, anti-HES1, and anti-EpCAM antibodies for control and L-685,458- or DAPT-treated tissues. Immunostaining was performed using DAB, and cellular nuclei were stained by hematoxylin. Scale bars = 100 μm. (**c**) Cumulative survival rate compared between GSI- (L-685,458 and DAPT) and control-treated NOD-SCID mice implanted with Huh7 cells. The survival rates of each of the five samples were compared and calculated using the log-rank *t*-test. **$P < 0.01$. (**d**) Liver cancer patients with high Jagged1 copy number variation (CNV) have poor survival. Overall survival data after surgical resection of liver cancer were divided into two groups (CNV <1.5 and ≥1.5). *P*-values were calculated using the log-rank *t*-test. The patients were followed up for up to 60 months after surgery. *$P < 0.05$

the GSIs had inhibited EpCAM production. Moreover, CK19 staining was significantly reduced in GSI-treated cases, indicating that the malignant characteristics of the hepatoma cells were controlled. The effect was more pronounced for DAPT ($p = 0.004$), and after a longer period of observation, there was a tendency for earlier death in the control cases (Fig. 6.1c).

Even in clinical liver cancer samples, the expression of AFP and Jagged1 showed a significant correlation, and amplification of the copy number of Jagged1 was associated with Jagged1 mRNA expression and poor survival after liver cancer surgical resection. CNV analysis of the Jagged1 locus in 110 liver cancer samples revealed that high CNV cases (≥1.5-fold, 65 cases) had lower survival rates ($p = 0.019$) than low CNV cases (<1.5-fold, 45 cases) (Fig. 6.1d).

From these experiments in vitro, in vivo, and in clinical samples, genomic amplification of Jagged1 was shown to contribute to mRNA expression in AFP-producing hepatoma cells, which activated the Jagged1-Notch signaling pathway in liver cancer and led to a poor outcome.

Notch Inhibition Is Effective for the Treatment of Liver Cancer

Our results indicated that inhibiting Notch signaling could be useful as an anticancer therapy that also targets LCSCs. However, the most famous Notch inhibitors, GSIs, have not been fully developed as an anticancer therapy due to gastrointestinal toxicity when used in Alzheimer's disease therapy (Doody et al. 2013; He et al. 2010). However, this strategy may be useful for developing novel anticancer agents in the future.

Notch signaling is enhanced in mouse cancer models, and inhibition of Notch signaling reduces tumor size. Moreover, Notch activation is reported to result in a more malignant phenotype, and as we also observed in our clinical samples, Notch upregulation is associated with poor outcome even after initial therapy such as surgery. Mouse models and human clinical samples have shown that the activation of Notch signaling results in a poor prognosis; thus, it would be a useful biomarker for aggressive types of liver cancer.

Experimentally, GSIs are useful for tumor suppression and prolonging survival in mouse liver tumor models (Gao et al. 2012; Shen et al. 2012; Suwanjunee et al. 2008). Several types of GSIs are being assessed in ongoing investigations and some have been shown to exhibit less toxicity; therefore, they may be useful as anticancer agents (Nakano-Ito et al. 2014). The pharmacological characteristics of GSIs indicate that there are some differences in the catalytic positions of gamma secretase at the transmembrane region, and several types of GSIs have been developed (Morohashi et al. 2006; Lu et al. 2014; Sato et al. 2006). One GSI shows less gastrointestinal toxicity and is in ongoing clinical trials (Doody et al. 2013; Shan et al. 2015). Besides reports of GSI treatments for liver cancer, there are some Notch-targeting therapies that use other clinically relevant drugs. Combination therapy with the anti-interleukin (IL)-17 antibody secukinumab and IL-35 blocks the Notch signaling pathway (Li et al. 2016). The effect of an Akt inhibitor on suppressing the proliferation of hepatoma cells by modulating the PI3-K/Akt and Notch pathways has been reported (Sokolowski et al. 2016). Thymoquinone exhibits a Notch-inhibiting effect with cell cycle suppression that is related to NICD expression (Ke et al. 2015). Moreover, this drug may be useful in combination with other anticancer therapies (Yang et al. 2018). It may be more effective to administer GSIs locally along with these therapies to reduce the adverse effects associated with GSIs.

Notch Signaling Enhances HBV Transcription

HBV induces liver cancer by chronic infection and inflammation. Viral proteins such as HBx induce the cancer-related signal by influencing p53 transactivation. However, the exact role of HBV infection in liver carcinogenesis is not fully understood. Many reports have discussed the role of HBV covalently closed circular DNA (cccDNA) in the nucleus of hepatocytes because the persistence of HBV cccDNA is a major obstacle to the elimination of chronic HBV infection and it is insensitive to antiviral drugs. Hepatocarcinogenesis is also related to HBV transcription from HBV cccDNA in hepatocytes, and we revealed that Notch signaling controls HBV cccDNA transcription in a ubiquitin-proteasome-dependent manner (Wang et al. 2019).

We found that E3 ubiquitin ligases regulated Notch signaling and HBV cccDNA levels. The E3 ubiquitin ligases ITCH and cooperator NUMB negatively regulate Notch1 by promoting NICD ubiquitination and degradation. We examined whether ITCH and NUMB were involved in the Notch signaling pathway and Notch-mediated upregulation of HBV cccDNA. Notch inhibition by Jagged1 shRNA and DAPT suppressed Notch1 and NICD, indicating diminished Notch pathway activity, and this was accompanied by elevated levels of ITCH and NUMB mRNA and protein, suggesting that ITCH and NUMB could negatively modulate the Notch signaling pathway (Fig. 6.2). These findings indicated that E3 ubiquitin ligases were essential for restricting Notch-mediated HBV cccDNA facilitation.

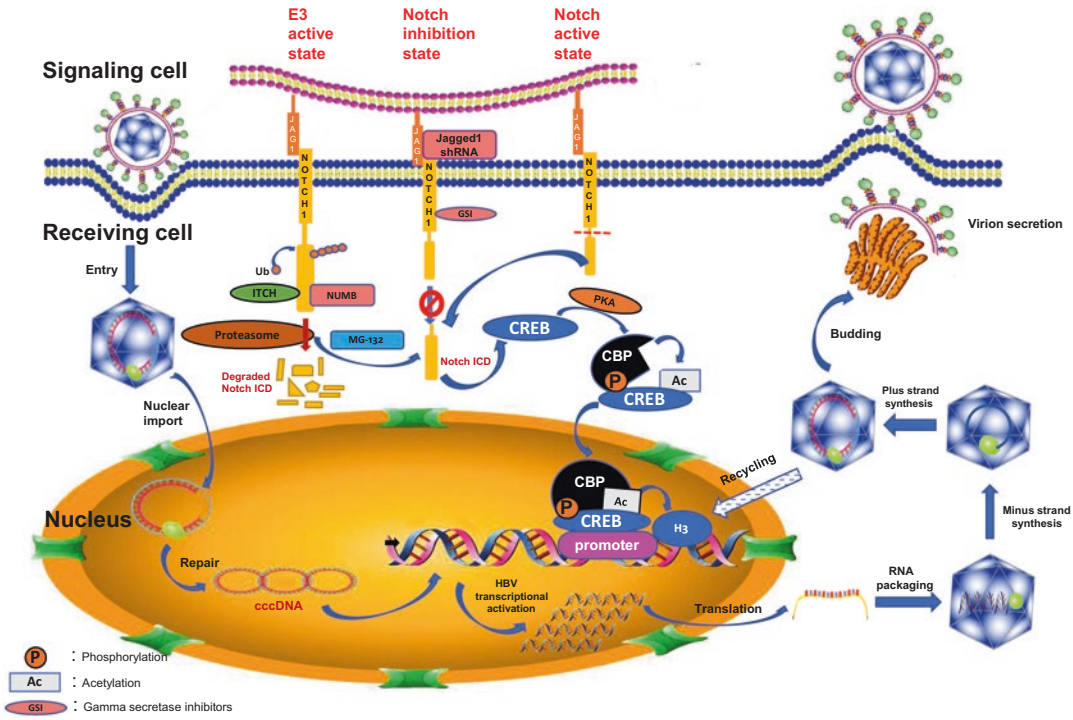

Fig. 6.2 Model summarizing the proposed Jagged1-Notch1-CREB signaling axis modulating HBV intrahepatic transcription and proteasomal degradation mediated by E3 ubiquitin ligases. Elements presumably connecting the Jagged1-Notch1-CREB-CBP circuit and Jagged1-Notch1-ITCH cascade are shown

HBV transcription is mediated by transcription factors and coactivators recruited to HBV cccDNA, including cAMP response element-binding protein (CREB) and CREB-binding protein (CBP). We analyzed whether the transcription factor CREB or its coactivator CBP played a role in the activation of HBV replication by Notch signaling. Our findings supported roles for the Notch-CREB (pSer133CREB)-CBP cascade in HBV intrahepatic replication and demonstrated the possible intranuclear mechanism by which Notch mediated HBV replication through the CREB-CBP signaling axis. CREB mediated the transcription of HBV cccDNA, and we evaluated relative gene expression in HBV-replicating cells treated with Jagged1 shRNA and GSIs.

Notch inhibition suppressed HBV cccDNA and CREB-related expression but increased ITCH and NUMB levels. The proteasome inhibitor MG-132 augmented HBV cccDNA levels, restored Notch and CREB expression, and inhibited ITCH and NUMB function. Increased levels of HBV cccDNA were observed after ITCH and NUMB blockage, even after treatment with the adenylate cyclase activator forskolin; a protein kinase A inhibitor had the opposite effect. Notch activation and E3 ligase inactivation were observed in HBV-positive cells in clinical liver tissue samples.

Our data demonstrated that the Notch signaling pathway played a crucial role in HBV cccDNA facilitation by activating the CREB/CBP cascade. In turn, this triggered the activation of HBV transcription, with blockage of this pathway possibly leading to a marked inhibition of HBV cccDNA via the upregulation of ITCH-NUMB in a ubiquitin-dependent proteasome-mediated manner.

Conclusion

Notch signaling abnormalities are present in various liver diseases including liver cancer and are affected by changes in the liver tissue microenvironment. These changes originate from hepatocytes and non-hepatocyte cells, including lymphoid cells, endothelial cells, stellate cells, and cholangiocytes. Notch inhibition-related anticancer therapy might be useful for hepatobiliary malignancies and be more effective in combination with existing anticancer drugs.

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Notch in Head and Neck Cancer

7

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Abstract

Head and neck cancer is a group of neoplastic diseases affecting the facial, oral, and neck region. It is one of the most common cancers worldwide with an aggressive, invasive evolution. Due to the heterogeneity of the tissues affected, it is particularly challenging to study the molecular mechanisms at the basis of these tumors, and to date we are still lacking accurate targets for prevention and therapy. The Notch signaling is involved in a variety of tumorigenic mechanisms, such as regulation of the tumor microenvironment, aberrant intercellular communication, and altered metabolism. Here, we provide an up-to-date review of the role of Notch in head and neck cancer and draw parallels with other types of solid tumors where the Notch pathway plays a crucial role in emergence, maintenance, and progression of the disease. We therefore give a perspective view on the importance of the pathway in neoplastic development in order to define future lines of research and novel therapeutic approaches.

Keywords

Notch pathway · Oral epithelium · Oral mucosa · Epithelial Notch · Oral cancer · Carcinoma · Squamous cell carcinoma · Cell-to-cell interaction · Intercellular communication

Abbreviations

ADAM	A disintegrin and metalloproteinase
AKT	Protein kinase B
APC	Adenomatous polyposis coli
CAF	Cancer-associated fibroblast
CCL	Chemokine (C-C motif) ligand
CCND1	Cyclin D1
cK	Cytokeratin
COX2	Cyclooxygenase-2
CRC	Colorectal cancer
CSC	Cancer stem cell
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DC	Dendritic cells
DLL	Delta-like
EGF(R)	Epidermal growth factor (receptor)
EMT	Epithelial-to-mesenchymal transition
FBXW7	F-box and WD repeat domain containing 7
GSI DAPT	Gamma-secretase inhibitors

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Hes	Hairy and enhancer of split-1
Hey	Hairy/enhancer-of-split related with YRPW motif protein 1
HIF1	Hypoxia-inducible factor 1
HNCs	Head and neck cancers
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
IL	Interleukin
ILC	Innate lymphoid cell
LAC	Lung adenocarcinoma
LNR	Cysteine-rich Lin-12/Notch repeats
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MALM	Mastermind-like
MET	Mesenchymal-to-epithelial transition
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
NCR	Natural cytotoxicity receptor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch intracellular domain
NK	Natural killer cells
NLS	Nuclear localizing sequence
Oct4	Octamer-binding transcription factor 4
OSCC	Oral squamous cell carcinoma
PTEN	Phosphatase and tensin homolog
PI3K	Phosphatidylinositol 3-kinase
RBPj	Recombination signal binding protein for immunoglobulin kappa J region
SCC	Squamous cell carcinoma
Shh	Sonic hedgehog
Sox2	Sex-determining region Y-box 2
STAT	Signal transducer and activator of transcription
TAMs	Tumor-associated macrophages
T-ALL	T-cell acute lymphoblastic leukemia
TCF	T-cell factor
TGFβ	Transforming growth factor-β
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
VEGF(R)	Vascular endothelial growth factor (receptor)
α-SMA	α-Smooth muscle actin

Introduction

Head and neck cancers (HNCs) represent a group of neoplastic diseases affecting different tissues and organs in the head and neck anatomical region. An estimated 5.5 million people are affected by HNCs with a poor prognosis of survival (379.000 deaths in 2015). More than 90% of HNC are carcinomas that mainly emerge from the epithelial wall of the oral cavity, although several other tissues, such as the nasal cavity, pharynx, larynx, and salivary glands, can also be affected (Sun et al. 2014; Mazur et al. 2010; Maliekal et al. 2008; Zagouras et al. 1995). Carcinogenesis is often triggered by chronic exposure to tobacco or alcohol, but it might also be associated with viral infections, commonly the human papillomavirus (HPV) infection (Rivera 2015; Bratman et al. 2016; Wang et al. 2019). Alcohol dissolves the lipid barrier in the external part of the epithelium and is metabolized into acetaldehyde, which in turn reacts with DNA molecules to induce damage (including adducts, single- and double-strand breaks, and point mutations) (Rivera 2015; Hunter et al. 2005). Similarly, tobacco-derived carcinogens (such as benzopyrenes, aromatic amines, and nitrosamines) covalently bind DNA, inducing gene mutations (Brennan et al. 1995). Finally, chemical exposure reduces the local immune surveillance, and local inflammation increases susceptibility to cancer development (Rivera 2015; Wang et al. 2019). Thus, chemically promoted DNA damage often results in an aberrant repair process, consequently causing uncontrolled proliferation and dysplasia of the epithelial layers (Hunter et al. 2005).

Conversely, infections with HPV require the presence of a preexisting wound, which allows the virus to reach the basal cell layer and integrate its dsDNA into the DNA of the host cell, ultimately exploiting the proliferative machinery of the epithelial tissue (Bratman et al. 2016; Bodily and Laimins 2011). Abrasion through mastication is a common cause of wound occurrence, and therefore the tongue and oral mucosa are the most common sites of cancer development (Ferlay et al. 2015; Ndiaye et al. 2014).

Less common neoplasms of the head and neck region include odontogenic tumors and tumors of the salivary glands. Salivary gland tumors represent 6% of all HNC and mainly occur in the parotid gland. A substantial risk factor for the development of a salivary gland tumor is exposure to ionizing radiation, which is typically used in radiotherapy. Odontogenic tumors are a heterogeneous group arising specifically in the jaw and are classified based on their peculiar ability to generate aberrant interconnection between ectomesenchyme and epithelium. Malignant odontogenic tumors are quite rare; however their benign counterparts are able to infiltrate surrounding structures, alter their architecture and function, and ultimately evolve into malignancy. Of the most common types of odontogenic tumors, the ameloblastoma and intraosseous carcinoma have epithelial origin and involve progression from altered odontogenic epithelium or an odontogenic cyst (Wright and Soluk Tekkesin 2017).

Histology of Craniofacial Epithelium

Epithelium lining the respiratory and digestive tract is often formed by squamous cells, of the simple or stratified type. Simple squamous cells line the air sacs of the lungs, but also of the heart, blood and lymphatic vessels and are characterized by a single layer of epithelial cells resting on the basal lamina. Stratified squamous cell epithelium is found in the oral and nasal cavity, but also in the esophagus, skin and vaginal walls. In contrast to the simple version of squamous epithelium, the stratified epithelium is composed of several overlapping layers of flattened cells, of which the first layer is in direct contact with the basement membrane. Cells are bound together by tight junctions with very limited or absent intercellular space. These structures allow resistance to constant abrasion and are normally found in areas where the physical barrier of the body meets the external environment. The continuous exposure to sheering forces induces the elimination of older cells from the external layers. As a result, stem and progenitor cells located in the deep basal layers undergo a proliferation stage and progress further in their maturation process, replenishing the outer layers of the stratified epithelium. Therefore, this type of epithelium faces a fast turnover, with cycles of stem cell activation, increased proliferation, and differentiation occurring in a finely coordinated manner.

In the head and neck region, the protective layers of a stratified squamous cell epithelium are the major protective structure of the tongue, oral mucosa, internal portion of the lips, larynx, and pharynx (Fig. 7.1). Similarly to specialized skin, the oral mucosa is additionally protected by a keratinized external layer. Cytokeratins (cK) are fibrous structural proteins abundant in epithelial cells, and the expression of specific keratins within a tissue determines both the type of cell and the function of the tissue. High levels of keratins increase the endurance of epithelium to mechanical stress and at the same time preserve hydration of its deeper layers. The basal epithelial cell layer, called the stratum basale, contains slow-cycling stem cells that are anchored to the basement membrane. The larger portion of the stratified epithelium is composed of several layers of proliferating progenitors that are generated from stem cells via asymmetric cell divisions and passively displaced toward the surface. When proliferative progenitors differentiate further, they acquire an elongated shape, leading to a tightening of the intercellular space by expression of modified desmosomes in the stratum granulosum. The most external layer of the squamous stratified epithelium is composed of maturing cells that produce and accumulate large amounts of protein aggregates containing keratin filaments. The large amount of aggregates promotes the collapsing and flattening of the corneocytes, while the synthesis of other proteins (involucrin, trichohyalin, and other small proline-rich proteins) continues. Proteins integrate into the plasma membrane, where they physically interact with membrane lipids (ceramides and cholesterol) to provide a waterproof barrier. When desquamation is induced by abrasion, desmosomes are degraded, and cells detach from the epithelial structure.

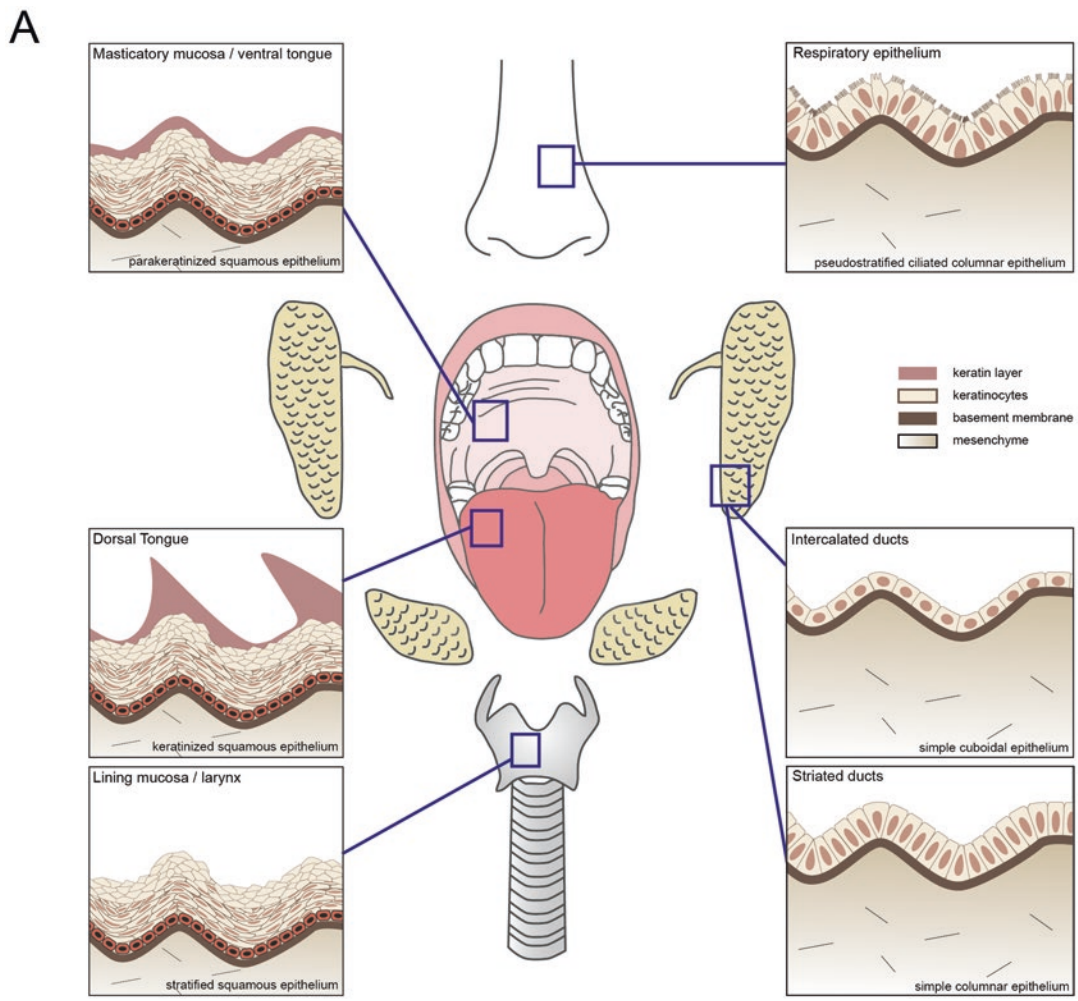

Fig. 7.1 Epithelia in the head and neck region. Various types of epithelial tissue line the walls of head and neck structures (A). Mesenchyme and epithelia are divided by the ECM-rich basement membrane (brown line), in direct contact with the first layer of immature keratinocytes. Depending on the type of cells found, epithelial tissue is classified as stratified squamous epithelium, cuboidal epithelium, and columnar epithelium. Epithelia exposed to a continuous mechanical stress, such as masticatory mucosa and tongue epithelium (upper and middle left panels), contain an external layer of deposited keratins to function as protective layer. Simple columnar epithelium is found in the striated ducts of salivary glands (right lower panel), while the intercalated ducts are mainly formed by cuboidal epithelium (right middle panel). A specialized form of columnar epithelium is found in the respiratory epithelium of the nasal mucosa, where cilia work as both a physical barrier to pathogens and are actively engaged in their removal via beating motion

More specialized epithelia characterize other head and neck regions, such as the ciliated pseudostratified columnar epithelium of the nasal cavity, trachea, and upper respiratory tract. Here, a single layer of ciliated columnar epithelial cells is positioned in direct contact with the basal lamina (Fig. 7.1). They possess cilia, projections extruding from the cell membrane, which help to trap particles brought in through respiration. Finally, the secretory function of the salivary glands is supported by cuboidal and columnar epithelia, which are differentially distributed within acini and ducti and are functionally important for the distinct levels of permeability of these structures to ions and water (Fig. 7.1) (Porcheri and Mitsiadis 2019).

Epithelial turnover varies greatly depending on the region analyzed (50–75 days in the skin, 4–14 days in the gut, 40–55 days in gingiva, and 25 days in the oral mucosa of the cheek) and correlates with epithelial maturation. Cancer treatment severely affects the dynamics of epithelium maturation and regeneration, as it interferes with mitosis regulation. Upon chemotherapeutic treatment, oral cancers patients often develop ulcers, experience painful conditions, and have difficulty in maintaining oral hygiene, eating, and drinking.

Epithelial Notch from Embryo to Cancer

Mammalian epithelial tissues derive from all three embryonic germ layers and form different anatomical structures (i.e., the epidermis derives from the ectoderm, inner epithelial walls from the mesoderm, and gut epithelium from the endoderm). The epithelium of the head and neck region has a heterogeneous nature from the beginning of its development. The oral mucosa is subdivided into epithelial walls composing palate, floor of the mouth, inner lips, and gingiva, all structures that are derived from the ectoderm. Conversely, the epithelium of the tongue is derived from endoderm and mesoderm (Winning and Townsend 2000; Rothova et al. 2012). Embryonic epithelial structures often constitute organ primordia and aid in shaping future functional units as a result of cell-to-cell interactions that coordinate specific molecular program activation, mechanical stimuli, and induction of maturation. During early embryonic stages (gestational week 4 in humans), neural crest cells migrate to the first and second pharyngeal arches to instruct the local epithelial structure in forming bone and cartilage of the face and neck but also pigments and cranial nerves. Once within the pharyngeal arch, neural crest cells surround a specific portion of the epithelium and give rise to odontoblasts, small bones of the middle ear, and thymic cells (Gilbert 2000).

The molecular mechanisms involved in the specification of craniofacial epithelium are largely unknown, but the essential role of cell-to-cell communication appears to play a major role in orchestrating maturation and dynamics of epithelial organs. In regard to intercellular communication, the Notch pathway plays an essential role in both inside-out and outside-in molecular activation (Siebel and Lendahl 2017). In mammals, four Notch receptors interact with specific ligands (three of the Delta-like type, Dll1, Dll3, Dll4, and two of the Jagged type, Jagged1, Jagged2) to regulate fate determination, survival, proliferation, and regulation of transcription (Fig. 7.2). Upon receptor interaction with the ligand, the intracellular portion of NOTCH is cleaved and transmigrates into the nucleus, where it recruits a transcription complex responsible for regulating the expression of specific target genes (among others of the Hes and Hey family: Hes1–7 and Hey1, Hey2, HeyL).

Specifically, the Notch target family of Hes genes is thought to be involved in the definition of a variety of head and neck structures, as mutations in these genes are involved in palate cleft, frontal bone agenesis, defects in cranial base formation, and proper size definition of lower and upper maxilla. These malformations are associated with an uncontrolled migration and positioning of the neural crest stem cells, which might utilize Hes1 as a regulator of local morphogenesis. Similarly, Hey1 was reported to be expressed in the branchial arches from early to late facial development, where a clear expression is confined to the epithelium of the nasal pit (Carbonell et al. 2018).

During embryonic development, several epithelial structures express Notch3 and Notch1 receptors, together with the ligands Jagged1 and Jagged2, and are involved in craniofacial morphogenesis (Zhu et al. 2017). Alagille syndrome patients have mutations in the gene coding for the Jagged1 ligand, resulting in facial hypoplasia and craniosynostosis. Depletion of the Jagged2 ligand results in altered tooth morphogenesis, mainly due to aberrant ameloblast differentiation and poor enamel deposition. Additionally, mutations of the Jagged 2 gene result in the abnormal fusion between palatal processes and the tongue, causing cleft palate. The Notch pathway has a specific

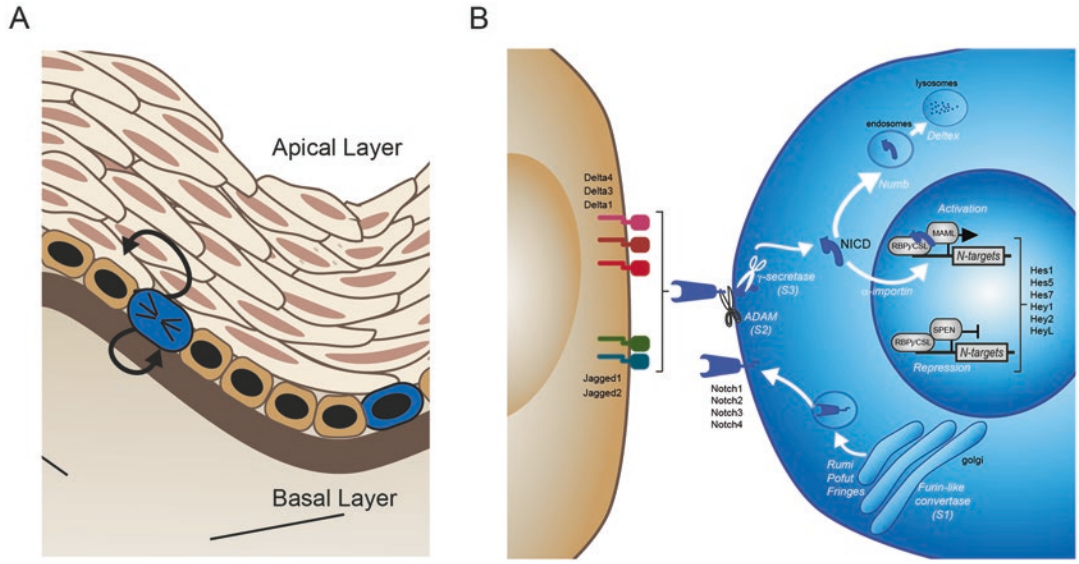

Fig. 7.2 The Notch signaling: controlling steps at a glance. The Notch signaling pathway is a complex pathway with various levels of regulation and fine-tuning. Initially, the immature NOTCH receptor faces a stepwise process of cleavage and glycosylation in the Golgi apparatus, before being exposed to the plasma membrane as a functional molecule. On the cell surface, it interacts with a member of the Delta or Jagged family present on the surface of the juxtaposed cell. Ligands present on the surface of the same cell exposing Notch participate in a more complex level of regulation of the pathway, known as *cis-inhibition* (not depicted, for reviews (Bigas and Porcheri 2018; Bray 2016)). Activation of the pathway occurs upon receptor-ligand physical interaction, when the receptor undergoes a series of cleavages for the intracellular release of its active intracellular domain (NICD), which then migrates to the nucleus to join the complex for the activation of transcription of Notch-target genes. In the absence of the NICD, the complex works as a repressor of transcription. The NICD is additionally regulated by its transport in the endosomes and final lysosomes degradation, under the control of specialized inhibitor of Notch activity (such as NUMB)

role in the regulation of tongue morphogenesis, where the crosstalk between Notch activity and the canonical Wnt signaling pathway directs the boundary formation between epithelium and mesenchyme, inducing the formation of the lamina propria during tongue organogenesis (Zhu et al. 2017).

Due to the involvement of Notch signaling in epithelial tissue definition and its role in balancing stem cell maintenance and differentiation, its activation is common to a variety of embryonic epithelial structures (Bigas and Porcheri 2018). In particular, the tongue epithelium shares strong similarities with the keratinized epithelium of the skin. From E8.5 in the mouse embryos, ectodermal cells activate the expression of cK5 and cK14 as differentiation markers of keratinocyte fate acquisition (Fuchs 2007; Koster and Roop 2007). Ectodermal cells at specific locations become columnar (epidermal placode), and interacting mesenchymal cells start to secrete extracellular matrix molecules that will provide an initial lamina upon which the epithelial stratification is built (Wessells and Roessner 1965; Stuart and Moscona 1967; Stuart et al. 1972; Holbrook et al. 1988; Kopan and Fuchs 1989). Ectodermal progenitors that acquire keratinocyte fate, start to express the differentiation marker p63 first, followed by keratin 14. Notch signaling is activated upstream of these markers, as blockage of the Notch signaling leads to a failure of keratinocyte differentiation in the mouse embryos (Tadeu and Horsley 2013; Candi et al. 2005).

Various events occurring during development are recapitulated during tumorigenesis, such as

the establishment of a supportive niche for undifferentiated cells, epithelial-to-mesenchymal transition, and change of balance between stem cell maintenance and differentiation (Fig. 7.3). The Notch pathway is involved in the regulation of common embryonic and oncogenic processes, and it is therefore not surprising that aberrant expressions of Notch receptors and ligands are hallmarks of several solid tumors, including head and neck tumors (Leethanakul et al. 2000).

Epithelial Notch has been specifically linked to the maintenance of cancer stem cells, the increase in invasion capabilities promoting epithelial-to-mesenchymal transition (EMT), and the constitution of a supportive tumor microenvironment (TME) (Fig. 7.3).

Notch in Cancer Stem Cells

Cells with characteristics of self-renewal and multi-fate differentiation have been identified in a variety of cancers, including colon, pancreatic, lung, and prostate carcinomas. Due to their low rate of proliferation, undifferentiated cells inside the tumor can escape chemotherapy and radiotherapy treatments and activate primordial programs for tissue homeostasis. Notch signaling is one of the major pathways involved in preserving undifferentiation of stem cells in both healthy and cancerogenic conditions (Bigas and Porcheri 2018) and has been specifically linked to cancer stem cell (CSC) self-renewal (Bolós et al. 2009; Wang et al. 2009a; Bolós et al. 2007). The Notch pathway increases stem cell survival in a variety of cancers (such as glioma and medulloblastoma (Fan et al. 2006)), and its activity results in induced de-differentiation of committed cells into more undifferentiated progenitors, as well as expansion of the stem cell pool by proliferation (i.e., mammary stem cells and mammospheres (Liu et al. 2005; Dontu et al. 2004)). A similar role of Notch is preserved in non-epithelial tumors, such as acute myeloid leukemia, where blocking the Notch pathway affects the survival of CD34+/CD38- undifferentiated populations (Gal et al. 2006).

Epithelial Notch operates as a stem cell keeper in a variety of tissues, with a few notable exceptions. In the head-neck region and specifically in the oral epithelium, stem cells located in the basal layer express the Notch1 receptor, although genetic depletion of Notch1 was reported to have limited effects on the maturation of normal mucosa. Oral epithelium lacking Notch1, displays unaltered morphology and expression of differentiated keratinocytes markers such as cK13 and cK15 (Barakat and Siar 2015; Sawangarun et al. 2018). In oral squamous cell carcinomas, the level of Notch expression correlates with tumor development and severity of dysplasia. In vitro assays based on head-and-neck squamous cell carcinoma (HNSCC)-derived spheres showed high expression of Notch1 and its direct role in regulating self-renewal. Overexpression of cleaved NOTCH induces the expression of classical stem cells markers, such as Oct4, Sox2, and CD44, while knockout of Notch1 inhibits tumor formation and increases sensitivity to chemotherapy (Lee et al. 2016).

As previously noted, the Notch signaling pathway has diverse, context-dependent functions. For example, in the adult gut, Notch preserves proliferation and undifferentiation via interaction with Dll1 and Dll4 ligands (Stanger et al. 2005). Notch1 upregulation results in maintenance of an undifferentiated state in colon cancer, mainly by interaction with Jagged1 and activation of Hes1 (Guilmeau 2012; Kazanjian and Shroyer 2011; Ueo et al. 2012; Peignon et al. 2011; Rodilla et al. 2009). In epidermal tumors, Notch works as a tumor suppressor, promoting differentiation of uncommitted progenitors in the hair follicle, sebaceous glands, and interfollicular epidermis (Nicolas et al. 2003; Okuyama et al. 2004). During the development of embryonic epidermis, Notch activity regulates the expression of p63, an important transcription factor involved in stem cell maintenance, and its upregulation in the basal layer prompts progression of differentiation (Tadeu and Horsley 2013; Estrach et al. 2008; Blanpain and Fuchs 2006; Lefort et al. 2007). Consistently, Notch deletion induces the development of spontaneous squamous cell

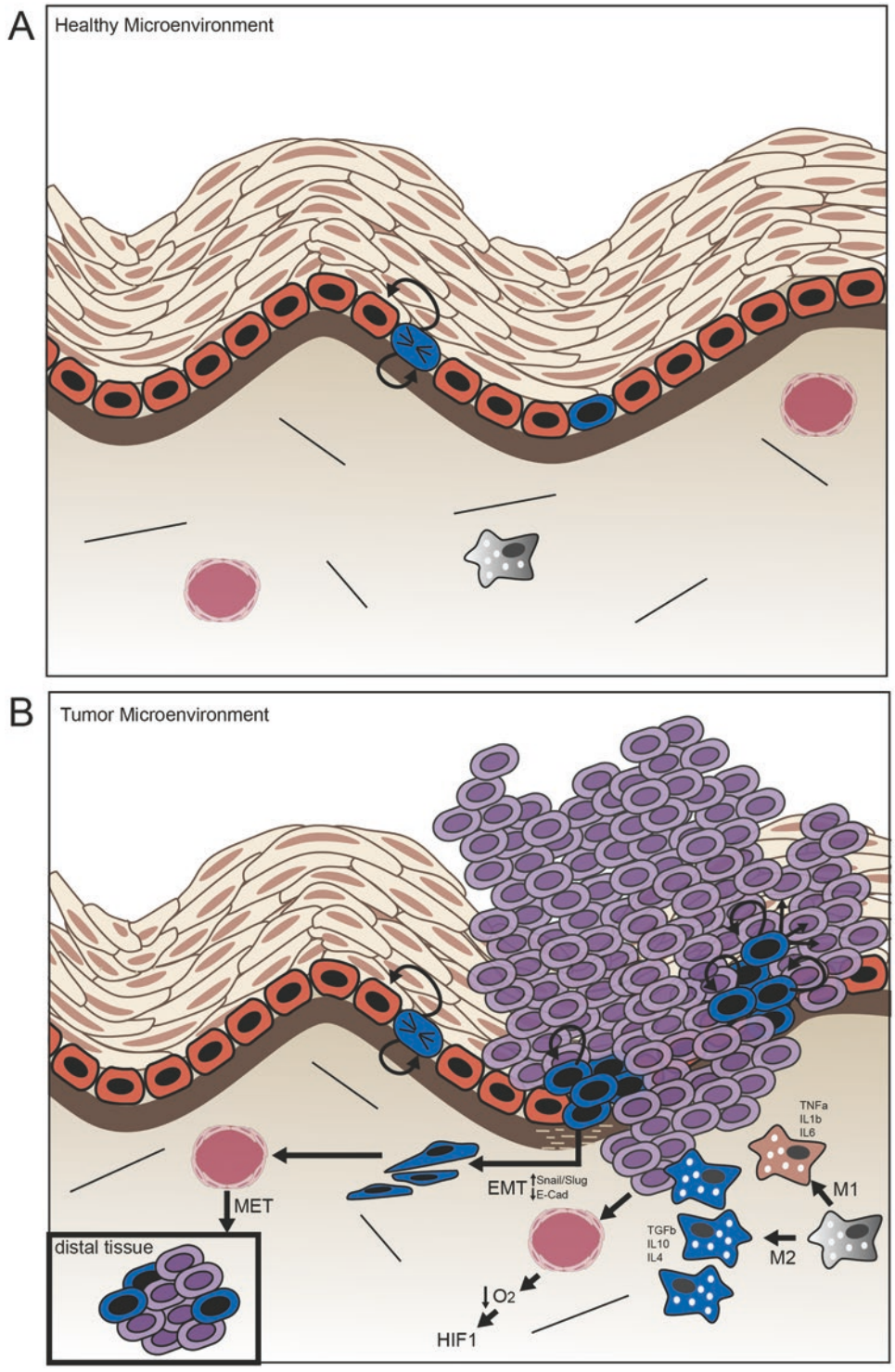

Fig. 7.3 Notch regulates major elements in the head and neck tumor microenvironment. The Notch pathway is involved in the maintenance of a healthy epithelium homeostasis (**a**) as well as central tumorigenic elements (**b**). It controls the balance between self-renewal and differentiation of stem cells in healthy conditions, as well as regulating the amplification of undifferentiated progenitors in the cancer core. It also participates in the process of EMT and its reverted MET, at the basis of invasiveness and metastases formation. Finally, it modulates the formation of an inflammatory milieu, inducing macrophage subtype specification and oxygen levels establishment in the tumor microenvironment. Depicted blue cells highlight the elements where Notch operates

carcinoma in the skin, probably via the Jagged1 ligand (Lefort et al. 2007; Estrach et al. 2006).

Due to the central role of Notch in preserving the more resistant core of cancer stem cells inside the tumor, the Notch signaling pathway represents a major therapeutic target. Radiotherapy treatments were shown to increase the activity of the Notch pathway, particularly in breast cancer and glioma stem cells. The radioprotective role of Notch might be linked with the Notch-dependent regulation of the AKT/PI3K pathway, which is specifically activated by the subpopulation of undifferentiated cells sustaining tumor survival (Phillips et al. 2006; Wang et al. 2010a).

Notch in Epithelial-to-Mesenchymal Transition

It is now widely accepted that epithelial cells can acquire a mesenchymal phenotype by the process of epithelial-to-mesenchymal transition (EMT). Upon tissue-reorganization stimuli, such as wound healing, embryonic development, and cancerogenic conditions, epithelial cells can undergo a remarkable change in morphology, modifying their genetic expression panel to acquire mesenchymal features. Specifically, they downregulate molecules involved in cell-to-cell junctions, such as E-cadherin and γ-catenin, destabilizing the epithelial structure and promoting cell migration (Christiansen and Rajasekaran 2006; Klymkowsky and Savagner 2009; Moreno-Bueno et al. 2008). An internal reorganization of the actin cytoskeleton together with the upregulation of mesenchymal markers (such as vimentin, fibronectin, α-smooth muscle actin, fibrillar collagen type I and type III, and fibroblast-specific protein-1) completes their change of fate (Christiansen and Rajasekaran 2006; Klymkowsky and Savagner 2009; Moreno-Bueno et al. 2008). Once the cell-to-cell interactions are disassembled, cells in transition start to produce and secrete matrix metalloproteases (MMPs) like MMP2, MMP3, and MMP9, increasing motility and invasion capabilities (Moreno-Bueno et al. 2008; Wang et al. 2010b). The Notch pathway has been found to be a major regulator in EMT, by regulating E-cadherin regulation of expression, the TGFβ pathway, and growth factor signaling. Repressors of E-cadherin genes (such as Snail and Slug) bind to the E-cadherin promoter to inhibit gene transcription (Becker et al. 2007). Notch directly regulates Snail and Slug, inducing their upregulation in epithelial cells and consequently downregulating E-cadherin expression, a crucial step in the early phase of EMT (Timmerman et al. 2004; Niessen et al. 2008). Additionally, Notch could control the expression of a hypoxic environment that would stabilize the Snail-1 protein (Sahlgren et al. 2008).

EMT is an essential event during wound healing and embryonic development and is Notch-dependent. For instance, during cardiac development, the Notch signaling pathway is expressed in endocardium and cardiac valve maturation, and both structures depend on EMT for completion. Similarly, embryos carrying Notch mutations or cardiac explants exposed to the Notch inhibitor DAPT, experience impaired EMT, which results in an aberrant cardiac valve formation (Timmerman et al. 2004).

In tumorigenic processes, downregulation of E-cadherin with a simultaneous increase of Snail expression is a hallmark of carcinomas (Brabletz et al. 2018). Activation of the EMT program can be triggered at the premalignant state of carcinoma, although their prognostic relevance in predicting the formation of new metastases remains to be elucidated (Hüsemann et al. 2008; Klein 2013). Interestingly, human samples and disease models display an incomplete form of EMT with epithelial and mesenchymal transcripts coexpressed in tumor cells (Mareel et al. 1992; Birchmeier and Behrens 1994). This partial activation of EMT allows tumor cells to convert back to an epithelial fate via a mesenchymal-to-epithelial transition (MET) when needed, increasing the level of plasticity of the invasive cancer (Fig. 7.3). Cells from the primary tumor can therefore utilize this great adaptability to establish metastases in distant tissues, increasing their heterogeneity and making their identification via transcriptional signature more challenging (Tsai et al. 2012). How EMT and MET contribute mechanistically to the spreading of the tumor remains to be understood.

Alterations to the surrounding microenvironment may help EMT establishment. Hypoxic conditions often present in poorly vascularized portions of carcinoma can contribute to the establishment of an EMT program. HIF1, a major hypoxia activator, indirectly represses the expression of epithelial key factors (such as E-cadherin) and thus promotes a mesenchymal differentiation (Krishnamachary et al. 2006; Esteban et al. 2006).

EMT is particularly important in conferring drug resistance (specifically to Taxol, vincristine, and oxaliplatin treatment (Wang et al. 2009b; Fuchs et al. 2008; Cheng et al. 2007; Sabbah et al. 2008)) and may be a useful therapeutic target to prevent the acquisition of an invasive phenotype and the development of metastases. Therefore, understanding the precise mechanism that governs EMT is an essential step for future cancer therapy, and Notch-dependent regulation of EMT offers a promising target for the molecular dissection of this process in human malignancies.

Notch in Tumor Microenvironment

A specialized tumor microenvironment (TME) sustains, nourishes, and protects tumor cells, which in turn can thrive supported by de-novo angiogenesis, a cooperative immune system, and a modified extracellular matrix. Solid tumors strongly rely on the intercellular communications with the surrounding environment for their maintenance. Cell-to-cell interactions become central in the regulation and generation of a cancerogenic milieu, and the Notch molecular pathway has been implicated in the regulation of various factors shaping the TME (Meurette and Mehlen 2018). As previously described, the expression of cadherins and integrins might directly depend on Notch regulation, being direct targets of the activated pathway. In parallel, Notch might also coordinate the activity of a subpopulation of resident cells (such as immune and endothelial cells) in the formation of a cancerous-prone environment.

Notch in Tumor Macrophages

TME is characterized by very specific immune infiltrates that can be exploited by the cancer itself for local release of tumorigenic factors. A specific subpopulation of bone marrow-derived monocytes give rise to tumor-associated macrophages (TAMs) that invade the aberrant tissue. Using the CCL2-CCR2 chemokine pathway, TAMs are recruited by the growing tumor mass whose constant release of trophic cytokines enhances angiogenesis and suppresses antitumoral T-cell response (Qian et al. 2011). TAMs have been identified in a variety of solid tumors of epithelial origin, such as breast, bladder, ovarian (Cassetta et al. 2016, 2019; Zhang et al. 2012), and head and neck cancers (Lee et al. 2014; Hu et al. 2016; Evrard et al. 2019). Macrophages are generally classified in M1 or M2 subgroups (Fig. 7.3). M1 macrophages are characterized by a pro-inflammatory phenotype, in that they release antitumoral cytokines such as TNFα, IL1b, IL-6, and CXC10, and their polarization is induced upon LPS or TNFα exposure. Tumor cells instead induce the acquisition of the M2 phenotype, characterized by the production and secretion of high levels of IL10, IL4, TGFβ, VEGF, and matrix metalloproteases to promote tumor survival and invasiveness. In addition to the regulation of the inflammatory milieu, TAMs might also be involved in controlling the EMT process, as suggested by in vitro models (Lee et al. 2018). Both subtypes of macrophages express the CD68 markers, while expression of M2 macrophages inside the tumor is specified by the expression of CD163. In oral and oropharyngeal carcinoma, high infiltrates positive for CD68 are a hallmark of the cancerogenic condition already present during the early stages of OSCC (Marcus et al. 2004). A correlative paradigm between poor survival rate in OSCC and high CD163 expression appears to be present in human samples where the TAM population localizes inside the tumor nest rather than in the tumor stroma (Fujii et al. 2012; He et al. 2014). Although the role of the M2 subpopulation seems to be the most relevant in shaping the tumor microenvironment, we cannot exclude a partial role of the M1

macrophages, as sub-distribution of the two populations might be relevant to understanding their mode of action. Additionally, a specific marker for M1 macrophages is still to be found, and their presence in a confined portion of the tumorigenic area cannot be excluded.

The Notch pathway has been largely associated with the induction of differentiation in both central and peripheral lymphoid organs, contributing to the development of the T- and B-lymphocytes. Notch might also be involved in the process of monocytic differentiation starting very early in the progenitor definition and fate acquisition. Gene expression barcoding revealed that Notch controls a myelomonocytic-specific gene signature via inhibition of transcription mediated by Hes1 (Klinakis et al. 2011). Additionally, macrophages and dendritic cells constitutively express members of the Notch family whose expression is regulated by the Toll-like receptor (TLR) (Palaga et al. 2008). TLR upregulates the expression of receptors, and Jagged1, Dll1, and Dll4 ligands directly promote Notch-pathway activation. During inflammation, cytokines like TNF and IL1b can induce the expression of Notch1, Notch4, and Jagged2 (Ando et al. 2003). As both inflammatory cytokines and TLR activate the NF-kB pathway, it has been postulated that this pathway functions as a molecular bridge in many systems, including cancer (Espinosa et al. 2010). In contrast with a pro-inflammatory fate acquisition driven by Notch activation, RBPJ depletion in TAMs blocks their differentiation and induces a previously inhibited cytotoxic T-cell response (Franklin et al. 2014). Therefore, it remains to be clarified whether blockage of Notch signaling involved in the pro-inflammatory induction can dampen the immune activation unfavorable for tumor growth. In epithelial cancers, Notch1 and Notch2 signaling through Jagged1 induces a TAM-anti-inflammatory phenotype (Liu et al. 2017). Interestingly, in head and neck squamous cell carcinoma, high levels of the Notch1 receptor are correlated with a high infiltration of $CD163^{+}$ TAMs (Franklin et al. 2014; Mao et al. 2018; Wang et al. 2010c).

Due to their supportive role in tumor growth and evolution, TAMs are under the spotlight as emerging therapeutic targets. Blockage of the CCl2/CCR2 interaction has been shown to be beneficial in human pancreatic ductal adenocarcinoma as it interferes with TAM recruitment (Nywening et al. 2016). Maturation of monocytes into M2 macrophages depends on IL6, which acts in synergy with other factors (such as COX2-dependent prostaglandin production and STAT3 activation). Current clinical trials are therefore focusing on inhibition of IL-6 and COX2 enzymes with promises to contrast lung and ovarian cancer progression (Edelman et al. 2008; Coward et al. 2011).

Notch in Tumor Angiogenesis

The tumor vasculature is an essential asset providing trophic support in solid tumor masses. Cancer cells secrete endothelial growth factors that sustain sprouting, migration, and rearrangement of vessels, in order to regulate the income of oxygen and nutrients enabling tumor growth. Notch signaling is a major regulator of physiological and aberrant angiogenesis, mainly through the Jagged1 or Dll4 ligands (Hellström et al. 2007). Vasculature dynamics depend on the coordinated activity of tip and stalk cells: high activation of Notch1 induces acquisition of the stalk phenotype, while low Notch activity is associated with tip-cell fate determination (Benedito and Hellström 2013). The difference in activity is directly associated with the type of ligand expressed on the surface of the different cell types, with tip cells expressing high levels of Dll4 and stalk cells expressing low levels of Dll4 and high levels of Jagged1 (Benedito et al. 2009). This in turn regulates the expression of the VEGF receptor and cell metabolism to drive directional sprouting and new vasculature formation (Benedito and Hellström 2013).

Tumors modify this balance to create the most suitable environment for their own growth, with high levels of Jagged1 inducing an increase in tumor vasculature and Jagged1 downregulation leading to decreased angiogenesis (Pedrosa et al.

2015). Metastatic tumors use the newly generated vasculature to enable spreading of mobilized cancer cells and may directly regulate endothelial cell quiescence (Sonoshita et al. 2011). Breast cancer cells, otherwise kept quiescent, proliferate in the presence of newly generated microvasculature derived from lung, brain, and bone marrow tumors, suggesting that a stable vasculature contributes to a dormant niche, while sprouting, activated endothelial cells are able to initiate metastatic growth (Ghajar et al. 2013). In line with these findings, high activity of the Notch3 receptor has been found in tumor vasculature (Lin et al. 2017). In aberrant conditions, Jagged1is overexpressed in cancer cells, blocking endothelial apoptosis driven by Notch3 and promoting local angiogenesis (Lin et al. 2017; Su et al. 2017; Zeng et al. 2005; Lu et al. 2013).

Regulation of tumor vascularization is also central in the establishment of a hypoxic microenvironment favored by a subset of carcinomas (i.e., lung, breast, kidney, and oral cancer) (Fig. 7.3) (Al Tameemi et al. 2019; Giatromanolaki et al. 2017; Chappell et al. 2019; Kujan et al. 2017; De Francesco et al. 2018).

Mammalian cells typically react to a reduced oxygen availability by activating transcription of HIF1 factor to trigger angiogenesis (Manalo et al. 2005). The two existing isoforms of HIFa (HIF1a and HIF2a) translocate to the nucleus upon binding with the HIF1b form, interact with the hypoxia-binding elements, and consequently promote the transcription of selected target genes. Activation of HIF has various implications in promoting tumorigenesis, including changes in metabolism and the production of oxygen radicals, maintenance of undifferentiation, and induction of a motile phenotype acquisition via EMT (Semenza 2012). Specifically, it directly correlates with advanced stages of oral cancer, and it has been proposed as significant prognostic marker (Fig. 7.3) (Qian et al. 2016). In hypoxic conditions both Notch activity and expression of Notch-dependent target genes are increased. Crosstalk of hypoxia and Notch signaling has been implicated in EMT and results in an increase of invasiveness in oral squamous cell carcinoma, although a full detailed mechanism of action remains to be described (Kujan et al. 2017; De Francesco et al. 2018; Yoshida et al. 2013; Wang et al. 2015).

Therefore, Notch signaling is involved in the regulation of normoxia and physiological vasculature growth, with important consequences for therapeutical application. Treatment with cetuximab showed broad effects on both Notch activation and HIF sensitivity, inhibiting tumor-induced angiogenesis in a murine model for HNSCC (Wang et al. 2015).

Notch in Carcinoma: When Context Matters

The Notch pathway is genetically altered in a large number of hematopoietic and solid tumors, resulting in aberrant activation or repression of the pathway. Different types of mutations interfere with receptor-ligand interaction, molecular regulation, gene targeting, and epigenetic regulations (Haines and Irvine 2003; Lei et al. 2003; Ntziachristos et al. 2014; Okajima et al. 2003).

In carcinomas, the role of epithelial Notch varies greatly depending on the organ affected (Kopan and Ilagan 2009; Dotto 2008; Dufraine et al. 2008). Emerging evidence suggests that the Notch signaling network is frequently deregulated in human malignancies, with upregulated expression of Notch receptors and their ligands in head and neck, cervical, lung, colon, and pancreatic cancer supporting the idea that Notch promotes cancer development (Miele and Osborne 1999; Miele et al. 2006). In a limited number of carcinomas, including skin cancer, human hepatocellular carcinoma, and small cell lung cancer, Notch signaling has been shown to be antiproliferative rather than oncogenic (Dotto 2008). It is therefore essential to establish the context of analysis to define the function of epithelial Notch as either oncogenic or antiproliferative (Kopan and Ilagan 2009; Dotto 2008; Dufraine et al. 2008). In the following paragraphs, we compare the role of Notch in a few relevant carcinomas, where its activity fluctuates significantly with its tumorigenic potential.

Head and Neck Cancer

Due to the high heterogeneity of the HNSCC and the different types of tissues that group under the same name, the exact function of the Notch pathway in this type of tumor remains to be clarified. All Notch receptors can be found highly expressed in HNSCC samples and OSCC, where they activate downstream signaling through Hey1 (Network 2015). Notch1 mutations are the second most common mutations found in head and neck carcinoma (HNSCC), suggesting an essential role of the pathway in the pathogenesis of the tumor. In an in vitro model of HNSCC, inhibition of Notch3 decreased sphere formation and proliferation in parallel with the inhibition of the activated target genes Hey1, cMyc, and CCND1 (Sun et al. 2014; Man et al. 2012).

In line with the oncogenic role of Notch1, its upregulation leads to resistance to chemotherapy treatments, and inhibition of Notch reduces the undifferentiated portion of cells in the HNSCC (Gu et al. 2010; Zhao et al. 2016). Dll4 overexpression can be found in subtypes of HNSCC and appears to have a role in vasculature reorganization and risk of metastases, consequently resulting in poor prognosis (Lin et al. 2010). Once activated, the Notch pathway increases the expression of target genes between normal mucosa and its dysplastic stage, with Hes1 and Hey1 being key players in the malignant condition (Sun et al. 2014). The upregulation of Hes1 seems to also correlate with an increase in undifferentiation or an amplification of the stem cell population, as demonstrated by self-renewal assay in sphere formation (Lee et al. 2012). Several studies identified high levels of Notch1 expression in HNSCC, especially when considering the subset of OSCC (Yoshida et al. 2013). Tongue cancer specifically displays an increased Notch3 expression that correlates with the degree of tumor progression, although cell proliferation does not appear to be altered (Zhang et al. 2011). High expression of Notch1 additionally correlates with high level of metastasis formation in patients with tongue cancer (Joo et al. 2009).

The Notch pathway is therefore relevant for HNSCC progression, although the exact mechanism of function remains to be identified.

Breast Cancer

Breast cancer is a form of cancer in which the Notch pathway may act as a both tumor suppressor and oncogene (Fu et al. 2010; Imatani and Callahan 2000; Jhappan et al. 1992). One of the first indications that Notch signaling may play a role in solid tumors came from experiments with mammary models developed after tissue infection with the mouse mammary tumor virus (MMTV). Integration of the MMTV genome next to the *Int-3* locus resulted in an activating mutation of *Notch4*, leading to the constitutive activation of the receptor and subsequent breast cancer development (Gallahan and Callahan 1997; Jarriault et al. 1995; Robbins et al. 1992). Recent observations indicate that Notch4 play a more specific role compared to other Notch receptors in breast cancer stem cells (Harrison et al. 2010) through signaling via other oncogenic pathways, such as Ras and Wnt (Ayyanan et al. 2006; Fitzgerald et al. 2000; Izrailit et al. 2013; Meurette et al. 2009; Weijzen et al. 2002).

Thus, Notch activation seems to play a protumorigenic role in breast cancer (Colaluca et al. 2008; Pece et al. 2004; Robinson et al. 2011; Xu et al. 2012). However, recent studies indicate that hyper-activation of NOTCH3 induce senescence in breast cancer cells and therefore have a detrimental effect on cancer development (Cui et al. 2013). This apparent divergence of results might be explained by the fact that mammary epithelial cells respond differently to different levels of activation of the Notch pathway (Mazzone et al. 2010). Consequently, different Notch receptors may have unique signaling outputs in mammary epithelial cells as well as producing alternative responses in different subtypes of breast cancers.

Colorectal Cancer

The intestinal epithelium possesses an unprecedented self-renewal rate that appears to be linked to a high susceptibility to malignant transformation (Legato et al. 1991; Miyaki et al. 2009). Notch signaling has been known to be involved in both the control of homeostatic self-renewal in stem cell populations and the development of

colorectal cancer (CRC) (Fre et al. 2005; Radtke and Clevers 2005; van Es et al. 2005). While mutations of *NOTCH* genes are rare, regulators of the pathway are often affected (including *FBXW7)*, resulting in an overall overexpression or uncontrolled activation of Notch signaling in CRC (Miyaki et al. 2009; Babaei-Jadidi et al. 2011; Camps et al. 2013; Sancho et al. 2010; Zhu et al. 2013). In addition, Notch activation has been linked to activation of Wnt signaling and Hippo/YAP signaling in CRC cells, although the various levels of crosstalk between these pathways are still not fully understood (Peignon et al. 2011; Rodilla et al. 2009; Fre et al. 2005; Tschaharganeh et al. 2013; Camargo et al. 2007; Kim et al. 2012; Kwon et al. 2011). In particular, Jagged1, expressed on tumor cells themselves or produced from endothelial cells, is thought to be a key ligand for Notch activation in CRC cells (Rodilla et al. 2009; Lu et al. 2013; Tschaharganeh et al. 2013). Another Notch ligand, DLL4, plays a non-cell autonomous role in CRC development, in large part by controlling the development of blood vessels necessary for tumor growth (Fischer et al. 2011; Ridgway et al. 2006). Expression of miR-34a in CRC stem cells may help to control Notch output and generate a bimodal Notch response (Bu et al. 2013). Finally, Notch signaling play a crucial role not only in the early stages of CRC development by controlling the fate of stem cells and cancer stem cells, but also at the later stages of tumor invasion and metastasis (Sonoshita et al. 2011).

Cutaneous Squamous Cell Carcinoma (cSCC)

Tumors arising from keratinized squamous epithelium can have different disease outcome; however they all derive from a disrupted differentiation of the basal progenitors, resulting in dysplastic epithelium and increased proliferation (Wang et al. 2011; Nowell and Radtke 2013). Cutaneous squamous cell carcinomas (cSCC) often occur as a result of exposure to UV radiation, which results in genetic aberration, mostly ending in TP53 loss. The Notch pathway has been found to be inactive in cutaneous SCC malignancies and instead works as a tumor suppressor under the control of p53 (Lefort et al. 2007; Wang et al. 2011). Notch1 and Notch2 mutations affecting the EGF repeats have been mapped in human cSCC and are linked to a dominant-negative phenotype (Rebay et al. 1991, 1993). Similarly, murine models of conditional Notch1 or Notch2 deletions result in structural defects and tumor formation, although the details of the molecular activation are not completely known (Demehri et al. 2009; Dumortier et al. 2010).

Lung Adenocarcinoma

Lung adenocarcinoma (LAC) is a major subtype of lung cancer (Licciulli et al. 2013; Westhoff et al. 2009; Zheng et al. 2013). In vitro studies initially identified the Notch signaling pathway as a promoter of LAC cell proliferation (Dang et al. 2003; Eliasz et al. 2010; Haruki et al. 2005). In parallel with these observations, in vivo modeling confirmed the relevance of the Notch pathway in preserving LAC development and maintenance (Licciulli et al. 2013; Allen et al. 2011; Maraver et al. 2012). Specifically, the NOTCH3 receptor is crucial in regulating the self-renewal of LAC tumor-propagating cells (Zheng et al. 2013). LAC cells express the Jagged2 ligand on their surface and support the metastatic potential of LAC stem cells (Yang et al. 2011). Thus, despite the absence of Notch mutations in LAC screenings, activation of Notch may be important in LAC growth, and Notch activity significantly correlates with the worsening of survival in lung cancer patients (Westhoff et al. 2009; Zheng et al. 2013; Hassan et al. 2013).

The squamous cell lung carcinoma (lung SCC) is the second major type of non-small cell lung cancer. Upon mapping of human-derived lung-SCC samples, several loss-of-function mutations were identified in the EGF-like repeats of the Notch1 receptor and result in a truncated, nonfunctional receptor. Therefore, in contrast to LAC, Notch may play a tumor suppressor role in the lung-SCC subtype of malignancy (Wang et al. 2011; Agrawal et al. 2012; Pickering et al.

2013; Proweller et al. 2006; Rothenberg and Ellisen 2012).

Thus, different subtypes of lung cancer display strikingly different roles for Notch signaling in cancer development, possibly depending on the type of cells involved, the crosstalk with other molecular pathways, or the fine-tuning of Notch activation in different biological context.

Notch as Therapeutic Target

Notch signaling is implicated in a variety of processes leading to cancer initiation, growth, and progression and has therefore been a focus for the development of novel therapies in the recent years.

As the Notch pathway requires a proteolytic cleavage by γ-secretase for the generation of its active intracellular form, small γ-secretase inhibitor (GSI) molecules have been developed to interfere with Notch1 activity (Fig. 7.2). Unfortunately, testing in animal models and clinical trials has revealed a high gastrointestinal toxicity due to the accumulation of goblet cells in the intestine upon Notch-dependent induction of differentiation (Aster and Blacklow 2012; Palomero and Ferrando 2009). Alternatively, a combination of treatment for GSI blockage and glucocorticoids mitigates intestinal side effects and controls goblet cell metaplasia (Real et al. 2009). Other proteolytic enzymes, such as ADAM10/17, participate in pathway activation and are used as target molecules for α-secretase inhibition (Fig. 7.2) (Zhou et al. 2006; Purow 2012). A promising approach involves specific blockage of the receptor-ligand interaction, for which a strong knowledge of the basic biological processes is necessary. Antibodies against Notch1 and Notch2 receptors protect the intracellular domain from its own cleavage, inhibiting the release of NICN1 and NICN2 active molecules (Fig. 7.2) (Wu et al. 2010). Importantly, the molecular specificity of these antibodies reduces the intestinal side effects, and, in particular, a blocking antibody against Notch1 showed promising results in inhibiting tumor growth (Funahashi et al. 2008). As the function of the Notch pathway strongly relies on the specific interaction between receptor and ligand, the efficiency of blockers varies from system to system (Hicks et al. 2002). Similarly to the blocking antibodies, several synthetic peptides have been developed to inhibit Notch activation. They are mainly used for basic research studies, although a blocker of the Notch-coactivator MALM1 was found to have useful applications in the treatment of several models of human T-ALL by interfering with cell proliferation and leukemia progression (Fig. 7.2) (Moellering et al. 2009). Finally, it might be of interest to alter the regulation of Notch turnover by interfering with its trafficking in the cancer cell secretory pathway (Fig. 7.2) (Ilagan and Kopan 2013; Krämer et al. 2013).

Anti-Notch therapies needs an overhaul when Notch works as tumor suppressor. In head and neck cancer, the heterogeneity of the tissues affected increases the level of complexity in predicting the exact role of the Notch pathway and which Notch receptor and ligand are most relevant to sustain the tumor. Mapping the expression and the level of activation of the pathway might be a valuable initial screening to determine the relevant molecules in each condition and their clinical interest. Finally, Notch agonists and antagonists could be used in combination with existing therapies to contrast tumor development.

Conclusions

To summarize, the Notch pathway plays an essential role in regulating major aspects of tumor emergence, maintenance, and evolution into a more aggressive phenotype. It is involved in preserving the cellular elements that sustain carcinoma, the formation of a supportive microenvironment, and their bilateral synergistic interaction. Although its effect varies greatly from system to system, Notch is central in head and neck malignancies, particularly in the most common and aggressive squamous cell carcinoma subtype. In order to improve our therapeutic approaches, we need a deeper molecular under-

standing of the function of the Notch pathway, including detailed knowledge of the pattern of expression in the heterogeneous population of tumor cells, genetic changes, transcript signatures, and fine-tuning of activity levels in a dynamic view, that takes into consideration the evolution of the disease over time.

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8 Notch Signaling and Human Papillomavirus–Associated Oral Tumorigenesis

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Abstract

The NOTCH pathway is critical for the development of many cell types including the squamous epithelium lining of cutaneous and mucosal surfaces. In genetically engineered mouse models, Notch1 acts as one of the first steps to commit basal keratinocytes to terminally differentiate. Similarly, in human head and neck squamous cell cancers (HNSCCs), NOTCH1 is often lost consistent with its essential tumor-suppressive role for initiating keratinocyte differentiation. However, constitutive NOTCH1 activity in the epithelium results in expansion of the spinous keratinocyte layers and impaired terminal differentiation is consistent with the role of NOTCH1 as an oncogene in other cancers, especially in T-cell acute lymphoblastic leukemia. We have previously observed that NOTCH1 plays a dual role as both a tumor suppressor and oncogene, depending on the mutational context of the tumor. Namely, gain or loss or NOTCH1 activity promotes the development of human papillomavirus (HPV)–associated cancers. The additional HPV oncogenes likely disrupt the tumor-suppressive activities of NOTCH and enable the oncogenic pathways activated by NOTCH to promote tumor growth. In this review, we detail the role of NOTCH pathway in head and neck cancers with a focus on HPV-associated cancers.

Keywords

Head and Neck Cancers · NOTCH · Human papillomavirus 16

Introduction

Head and neck squamous cell carcinomas (HNSCCs) develop from the squamous mucosal lining of the aerodigestive tract primarily encompassing the nasopharynx, oral cavity, oropharynx, larynx, and hypopharynx (Argiris et al. 2008). HNSCC is the seventh most common type of cancer, constituting 5% of the all the cancers worldwide (Siegel et al. 2019), and causing more than

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50,000 new cases and 12,000 deaths each year in the United States (Argiris et al. 2008). According to the Surveillance Epidemiology and End Results (SEER) database, the 5-year survival rate of patients with HNSCCs approximates 50–60% (Siegel et al. 2019). Despite substantial progress in surgical and radiotherapeutic techniques as well as the introduction of chemotherapy and immunotherapy agents alone or with radiotherapy, survival has only been modestly improved over the past 30 years (Rampias et al. 2014).

HNSCCs are divided into two categories: human papillomavirus (HPV)–associated cancers and HPV-negative cancers. Currently, HPV-associated cancers primarily arise in the oropharynx, and account for 70% of cancers in this site driving the recent increase in oropharyngeal cancer incidence (Chaturvedi et al. 2011). The HPV-associated oropharyngeal cancers occur more often in younger, nonsmoking patients and are associated with better survival outcomes compared to HPV-negative patients (Ang et al. 2010). Although HPV-positive patients have substantially better prognosis, approximately 20% of patients still fail to therapy, indicating the unmet need to understand the biology underlying HPV carcinogenesis and metastasis.

HPV HNSCCs also comprise a subset of virally induced cancers, many of which regulate members of the Notch pathway (Vazquez-Ulloa et al. 2018). In fact, HPV oncogenes disrupt NOTCH transcription of differentiation-related genes, which may promote carcinogenesis. Here, we will discuss the intersecting roles of HPV and the NOTCH pathway during epithelial differentiation and carcinogenesis.

HPV Life Cycle Promotes Viral Replication in Differentiating Keratinocytes

All papillomaviruses genomes exist as circular double-stranded DNA episomes of approximately 8000 base pairs in the nucleus of host cells. The HPV genome contains eight open reading frames falling into three major regions: (1) an early gene region, (2) a late gene region, or (3) a long control region. These regions are separated by two polyadenylation sites (Stoler 2000; Doorbar et al. 2015). The six early genes, E1, E2, E4, E5, E6, and E7, encode proteins necessary for viral replication and, as an unintended consequence, cellular transformation. The two late genes, L1 and L2, encode structural proteins of the virus necessary for viral capsid formation (Graham 2010). The 1000–base pair noncoding region is essential viral DNA replication by containing elements that regulate the spatiotemporal differences in early and late gene expression.

HPV replication is intertwined with keratinocyte differentiation and characterized by three distinct phases of replication (Fig. 8.1). First, HPV establishes infection by gaining access to the proliferating stem cells in the basal keratinocyte layer via wounds or microabrasions that disrupt the epithelial layer. After infection of basal keratinocytes, HPV initiates "establishment replication" to generate 50 to 100 viral DNA copies in an episomal form that reside in the undifferentiated basal cell reservoir.

Of the HPV genes, the E6 and E7 genes are best known to contribute to oncogenesis (Fig. 8.2). The E6 protein binds the tumor suppressor protein p53 along with the cellular E3-ubiquitin ligase E6-AP in order to target p53 for ubiquitination and subsequent proteasomal degradation. E7 binds to retinoblastoma (Rb), also a tumor suppressor, to facilitate the release of E2F family of transcription factors.

The second phase of HPV replication, the early phase, begins upon epithelial differentiation where viral genomes are amplified in the more differentiated suprabasal layers. Viral replication is regulated by the six early genes. E1 and E2 promote viral genome replication as E1, a virus-specific helicase, aids the unwinding of viral DNA, and E2 regulates the expression of viral and cellular genes necessary for replication. E6 and E7 interact with p53 and pRb, respectively, to control apoptosis, differentiation, and cell cycle which is necessary for viral replication in differentiating cells (Blanpain et al. 2006). E5 is primarily expressed in high-risk HPV subtypes and plays an important but less recognized role in cellular transformation and immune escape.

The final phase of HPV replication, the late phase, involves virion packaging, assembly, and

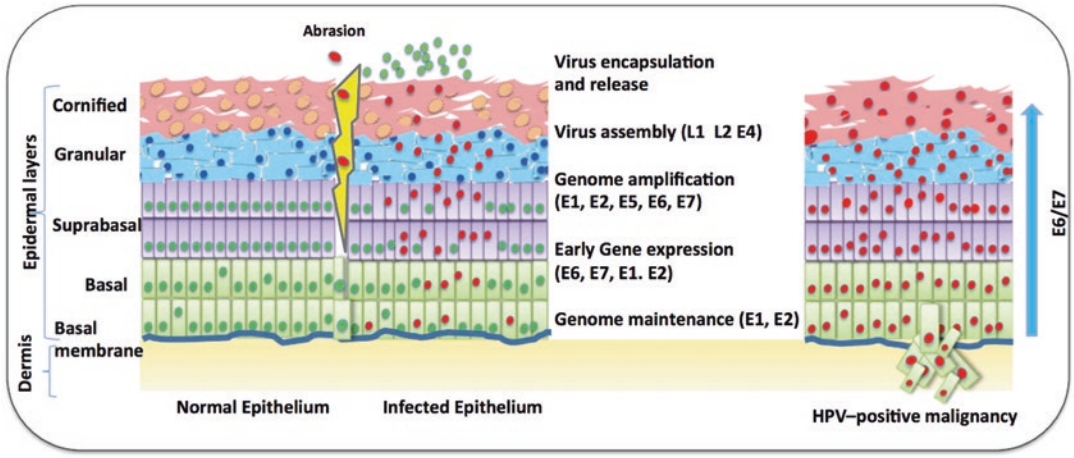

Fig. 8.1 The HPV life cycle. (Left panel) HPV infection occurs as epithelial disruptions enable the virus access to the basal keratinocyte layer. Here, early genes are expressed with disrupt differentiation to enable viral replication. Viral particles are release at the epithelial surface where terminal differentiation occurs. (Right panel) HPV infection and aberrant differentiation and proliferation lead to HPV-associated cancers

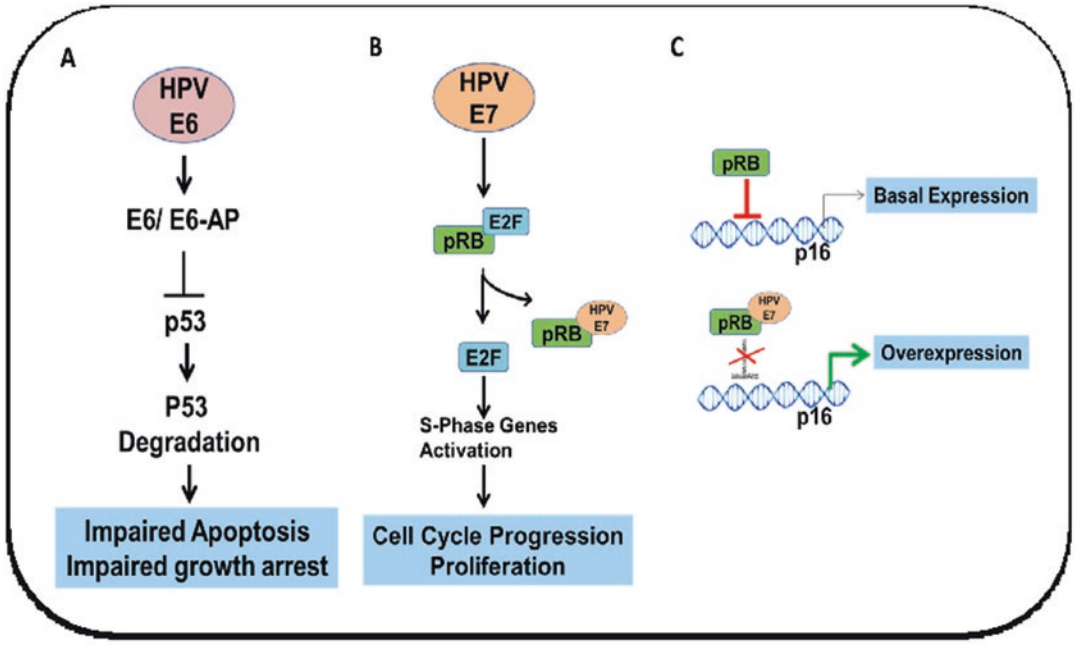

Fig. 8.2 Function of the HPV oncogenes E6 and E7. (**a**) HPV E6 oncogene impairs the function of p53 by shuttling p53 towards degradation. (**b**) HPV E7 oncogene impairs the E2F/pRb complex and allowing free E2F to enter continuous cell cycle progression to promote aberrant cellular proliferation. (**c**) pRB regulates p16 expression and inhibitory E7 viral protein relieves the repression by pRB

release. E4 plays a crucial role in packaging the viral genome, and the two late genes (L1 and L2) comprise the virus capsid required for the production of mature viruses. This occurs in terminally differentiating keratinocytes which release HPV virions for further infection. Thus, the HPV genome is designed to disrupt keratinocyte differentiation in order to facilitate viral replication and release.

NOTCH Pathway Initiates Spinous Differentiation of Basal Keratinocytes

The stratified squamous epithelium consists of keratinocyte cells layered upon a basement membrane. The keratinocyte cells most proximal to the basement membrane comprise the basal layer. The basal layer, along with the hair follicles in cutaneous epithelium, contains the stem cell compartment from which keratinocytes differentiate. The initial steps of epithelial differentiation involve the keratinocytes leaving the basal layer to the suprabasal layers, traversing from the spinous layer to the granulosum layer, to the lucidum layer, and finally to the corneal layer. As keratinocytes traverse each layer, they further differentiate, acquiring expression of different keratins and other cytoskeletal and other structural proteins as well as undergoing nuclear involution and loss of reproductive capacity.

Notch was initially described in the fruit fly *Drosophila melanogaster* in the early twentieth century as a phenotype where female flies developed "notches" in their wings (Mohr 1919). The Notch pathway is highly conserved signaling pathway regulated by cell–cell communication. In humans, Notch family members include NOTCH1, NOTCH2, NOTCH3, and NOTCH4. The NOTCH proteins are Type I transmembrane receptors composed of a large extracellular domain, a single transmembrane domain, and an intracellular domain that executes the NOTCH transcriptional program (Fig. 8.3). The NOTCH receptor recognizes five ligands: the Delta-like ligands DLL1, DLL3, and DLL4 and the Serrate-like ligands Jagged1 and Jagged2. NOTCH signaling is initiated when cell surface ligands, including Delta-like 4 and Jagged family members, on one cell bind to NOTCH receptors on an adjacent cell resulting in transmembrane cleavage of NOTCH and the release of the intracellular (NICD) domain (Mumm and Kopan 2000).

The Notch family functions in a cell and context-specific manner to regulate cell-fate determination and differentiation (Bray 2016). In many tissues, such as the hematopoietic and pancreatic organs, Notch activation has been shown to maintain stem cell potential and inhibit differentiation. However, in other contexts, including squamous epithelium, Notch activity induces the exit of keratinocytes from the stem cell compartment via two ways. First, localized NOTCH expression commits cells to a "transient amplifying" phenotype where cells retain limited proliferative potential but are committed toward a terminal differentiation program (Lefort and Dotto 2004; Lowell et al. 2000). Alternatively, the Fuchs group demonstrated that mice overexpressing the constitutively activated NICD1, the constitutively active truncated C-terminal domain of Notch1, led to expansion of the spinous layer and induction of differentiation-related genes specific for cells in the spinous layer (Blanpain et al. 2006). The expression of these spinous-related genes was dependent on the expression of the Notch1 target gene Hes1. Notch differentiation programs are likely dependent on the polarity of the mitotic spindle as cells unable to initiate asymmetric cell division, defined by the orientation of mitotic spindles to the basal layer, and displayed impaired Notch1 signaling and spinous differentiation (Williams et al. 2011). Thus, in contrast to other organ systems where the NOTCH pathway preserves the stem cell compartment, in the skin and mucosal surfaces, the NOTCH pathway promotes epithelial differentiation via distinct mechanisms.

NOTCH signaling programs are activated by trans-receptor–ligand interactions on adjacent cells, resulting in the successive cleavage of NOTCH proteins. NOTCH is first cleaved by TNFα converting enzyme (TACE), a member of the ADAM-17 family of metalloprotease (van Tetering et al. 2009). The subsequent cleavage results from a γ-secretase–presenilin complex (Meng et al. 2009), resulting in the release of the Notch intracellular domain (NICD), a functional, active C-terminal NOTCH fragment. The NICD translocates to the nucleus and binds via its RAM and ankyrin domains to the DNA-binding transcription factors CBF or RBP-JK recruiting coactivators such as Mastermind like-1 (MAML-

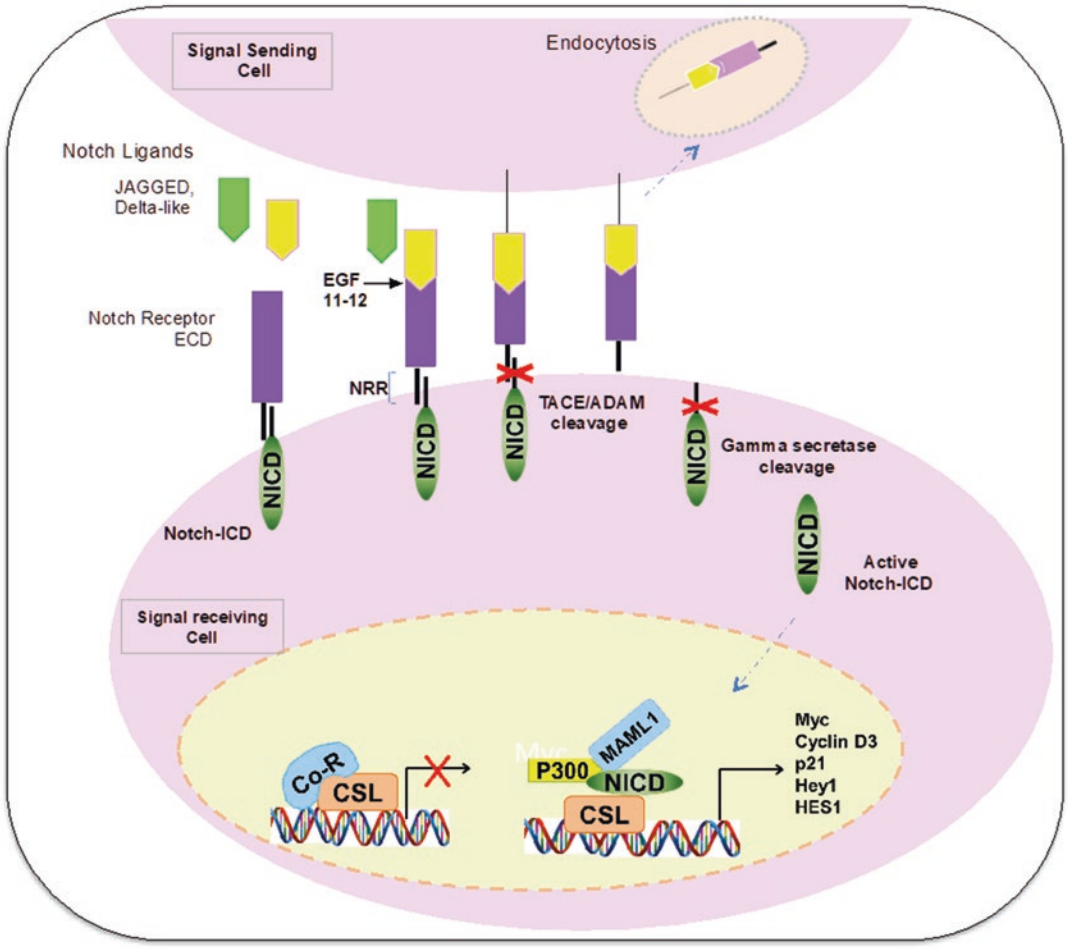

Fig. 8.3 Notch signaling pathway. Notch ligand on one cell induces a series of proteolytic cleavage events in the Notch receptor on an adjacent cell. These cleavage events release the Notch intracellular domain (NICD), which translocates to the nucleus to activate the transcription of Notch target genes together with CSL and Mastermind-like protein (MAML)

1) and p300/CBP (Hansson et al. 2009; Wu et al. 2000; Wu and Griffin 2004). In the absence of NICD, CBF-1 represses gene expression by binding the histone deacetylase complex SMRT–sin3 and HDAC-1. Binding of NICD to CBF-1 displaces the repressor complex and recruits nuclear coactivators, such as MAML1 and histone acetyltransferases (Hansson et al. 2009; Wu et al. 2000; Wu and Griffin 2004; Guruharsha et al. 2012). The conversion of CBF-1-NICD from a transcriptional repressor to a transcriptional activator results in the expression of target genes including hairy/enhancer of split (HES) gene family and HEY subfamily members (Rettig et al. 2015). In addition, CBF-1-NICD also induces the expression of cell cycle–related genes, p21, p27, E2F, and transcription factors NF-κB and peroxisome-proliferator-activated receptor transcription factors that subsequently execute pro-survival functions toward carcinogenesis (Brimer et al. 2012; Rangarajan et al. 2001; Suman et al. 2014).

HPV Carcinogenesis Occurs via Disruption of p53 and pRB

Approximately 200 serotypes of HPV exist and are classified into five genera (α, β, γ, μ, ν) based on DNA sequence similarity and tissue tropism (Van Doorslaer et al. 2013; Moody 2017). Alpha-papillomaviruses (α-HPV) infect mucosal tissues, whereas β-, γ-, ν-, and μ-papillomaviruses infect cutaneous tissues (Rautava and Syrjanen 2012). Overall, mucosal tropic HPVs infect the anogenital tract, upper digestive tract causing HNSCC, cervix cancer, vaginal and vulvar cancer, and anal cancer. Mucosal types can be subdivided into low-risk and high-risk serotypes that are associated with differing degrees of oncogenic potential. Low-risk serotypes are associated benign genital lesions and include HPV 6 and 11 serotypes. High-risk serotypes are associated with cancers and include HPV16, 18, 31, 33, 45, 52, and 58 serotypes. Of these high-risk subtypes, HPV 16 and 18 are most commonly associated with cervical cancer and HPV 16 is almost exclusively associated with oropharyngeal carcinomas (Stoler 2000; Kreimer et al. 2005; Miralles-Guri et al. 2009). Unlike α-HPVs, most of the β-HPV and γ-HPVs infections are asymptomatic in immunocompetent individuals without any clinical manifestations of disease (Gottschling et al. 2009; Nindl et al. 2007).

Classically, the initial steps of HPV carcinogenesis occur via the integration of high-risk HPV episomal DNA into the host-cell genome. While HPV-associated cancers can arise without integration of the viral genome, this is usually less frequent. Analysis of The Cancer Genome Atlas (TCGA) demonstrated that more than 80% of cervical cancers displayed integrated viral genomes (Cancer Genome Atlas Research N, Albert Einstein College of M, Analytical Biological S, Barretos Cancer H, Baylor College of M, Beckman Research Institute of City of H, et al. 2017). By contrast, the HPV genome is integrated in oropharyngeal cancers at slightly lower rates, approximating 70% (Parfenov et al. 2014; Vojtechova et al. 2016). Although several groups have suggested differences in outcomes in patients with episomal versus integrated HPV genomes, the data is overall conflicting where one cannot draw a consensus opinion. The integration of the HPV genome usually disrupts the E2 protein, which regulates the transition of early gene expression to late gene expression in differentiating keratinocytes. This results in a loss of the negative feedback controlling E6 and E7 expression and, consequently, the persistent expression of E6 and E7 and the resulting disruption of the tumor suppressors p53 and pRB, respectively (Collins et al. 2009).

Dysfunction and inactivation of p53 and pRb are the classical, initial steps for the development of cancers in multiple tissue types (Fig. 8.2). In HPV-associated cancers, E6 disrupts p53 by forming a trimeric complex with p53 and the cellular ubiquitin ligase E6-AP protein (Huibregtse et al. 1995) leading to the ubiquitination and rapid proteasomal degradation of p53 (Talis et al. 1998; Munger et al. 2004). The targeting of p53 by E6 prevents the activation of cell death pathways that would normally be activated by abnormally proliferating cells. By contrast, E7 canonically disrupts the Rb family of proteins including Rb, p107, and p130, to induce abnormal cellular proliferation. E7 contains an LXCXE motif that binds to the pocket of Rb to disrupt the sequestration of E2F family members from the nucleus. Once E7 binds to Rb, E2F is freed to migrate to the nucleus and to induce expression gene regulating cell cycle progression and, as a consequence, genomic instability (Ghittoni et al. 2010).

HPV and NOTCH in HNSCCs

The TCGA and other massively paralleled sequencing efforts to elucidate the mutational changes in HNSCCs and other squamous cell cancers have identified both cellular and viral drivers of carcinogenesis (Cancer Genome Atlas Research N, Albert Einstein College of M, Analytical Biological S, Barretos Cancer H, Baylor College of M, Beckman Research Institute of City of H, et al. 2017; Cancer Genome Atlas 2015; Gillison et al. 2019; Seiwert et al. 2015) (Table 8.1). Previous studies have shown that

Table 8.1 Molecular identification of genes and proteins that implicates a role in HPV-positive and HPV-negative head and neck squamous cell carcinoma

Gene	Role	Outcome
TP53	Tumor suppressor gene	60–80% of HSNCC has mutated p53
PIK3CA	A catalytic subunit of PI3K effects on metabolism, proliferation, and cell survival	PIK3CA mutations in 8% HNSCC samples, 21% of HNSCC samples
EGFR	Transmembrane receptor and cellular homeostasis	90% of HSNCC showed overexpression Negative prognostic factor after radiotherapy
FGFR	Role in cellular differentiation, migration, and angiogenesis	FGFR1 amplification or mutation is seen in 10% of HPV-negative HNSCC and FGFR3 mutations or fusions occur in 11% of HPV-positive HNSCC
Cyclin D1	Protooncogenes, regulates cell cycle progression G1-S	TCGA study showed 28% of HNSCC has CCND1 amplification, with 32% (77/243 in HPV-negative and 6% (2/26) in HPV-positive samples Resistance to Cisplatin
PTEN	Tumor suppressor genes play a role in apoptosis	11% of HPV-positive HNSCC and 5% of HPV-negative HNSCC
C-MET	EMT and invasion	MET–HGF axis as therapeutic target in HNSCC
MMPs	Degrade basement membrane of ECM and helps in cancer progression, invasion	Overexpression of MMPs-2, 8, and 13; involved in lymph node metastasis and chemotherapy resistance
NOTCH	Tumor suppressor gene as well Oncogene	14–15% of HNSCCs has inactivating mutations 32% has activating mutations
P16	Tumor suppressor regulates cell cycle progression	50–80% of HNSCC has loss of p16
HIF-1α	Involved in angiogenesis and EMT	Roles in chemoresistance, radio-resistance, and poor prognosis
ERBB	Tyrosine kinase	Amplification and mutation are seen in 4% HPV-negative and 3% of HPV-positive HNSCC Afatinib and dacomitinib (irreversible pain inhibitors are on clinical trial)

mutation of genes *TP53, CDKN2A, PIK3CA, EGFR, CCND1, PTEN*, and *HRAS*, either by gain or loss of function together with *FBXW7, NOTCH1, IRF6*, and *TP63*, causes dysregulation of signaling pathways and chromosomal abnormalities that are responsible for pathogenesis of HNSCC (Leemans et al. 2011; Stransky et al. 2011; Pickering et al. 2013; Agrawal et al. 2011). Of note, HPV-associated HNSCCs have a genetic landscape that is distinct from HPV-negative HNSCCs. Globally, HPV-negative HNSCCs display approximately a two-fold greater mutational burden that HPV-positive tumors, which was independent of smoking status (Stransky et al. 2011). Furthermore, the presence of mutations in TP53 was inversely associated with HPV tumor status as no HPV-associated cancers had TP53 mutations while 78% of HPV-negative cancers contained a TP53 mutation (Stransky et al. 2011). Furthermore, HPV-negative HNSCCs are more likely to have higher expression of EGFR and chromosomal aberrations in 3p, 9p, and 17p (Munger et al. 2004; Benson et al. 2014; Braakhuis et al. 2004; Kumar et al. 2008). In addition, we have shown HPV oncogene expressing HPV-positive autochthonous oral tumors grew faster and gained expression of MCM7 as compared to HPV-negative tumors (Zhong et al. 2014).

The TCGA along with other sequencing efforts identified disrupting mutations or loss of NOTCH1 as a frequent event in head and neck cancers (Cancer Genome Atlas 2015; Agrawal et al. 2011). Agarwal et al.

demonstrated that NOTCH1 was frequently mutated in HNSCCs in which 40% of these mutations were predicted to truncate and inactivate the NOTCH1 gene product (Agrawal et al. 2011). Stransky et al. identified mutations in *NOTCH1, IRF6,* and *TP63* genes in 30% of HNSCC patients (Stransky et al. 2011). Similarly, the TCGA demonstrated frequent mutations in differentiation-related genes including NOTCH1, TP63, FAT1, and AJUBA (Cancer Genome Atlas 2015). Similarly, we observed Notch1 was frequently mutated using a transposon-based insertional mutagenesis as a functional screen to identify cellular genes responsible for autochthonous HPV-positive tumors (Zhong et al. 2015).

The Interaction of NOTCH Pathway During Viral Carcinogenesis

HPV oncogenes likely modulate the NOTCH pathway and vice versa in HPV-associated cancers. Several reports have shown that several HPV serotypes, including HPV5, HPV8, and β-HPVs impair NOTCH activity by manipulating the NOTCH-associated transcriptional machinery (Fig. 8.4). The E6 of β-HPVs directly binds to the MAML1 and interferes with the interaction of MAML and NICD, resulting in the loss of expression of NOTCH target genes HEY and HES (Rampias et al. 2014; Brimer et al. 2012; Meyers et al. 2013). HPV cancers have also been shown to directly downregulate NOTCH expression in order to inhibit the NOTCH pathway.

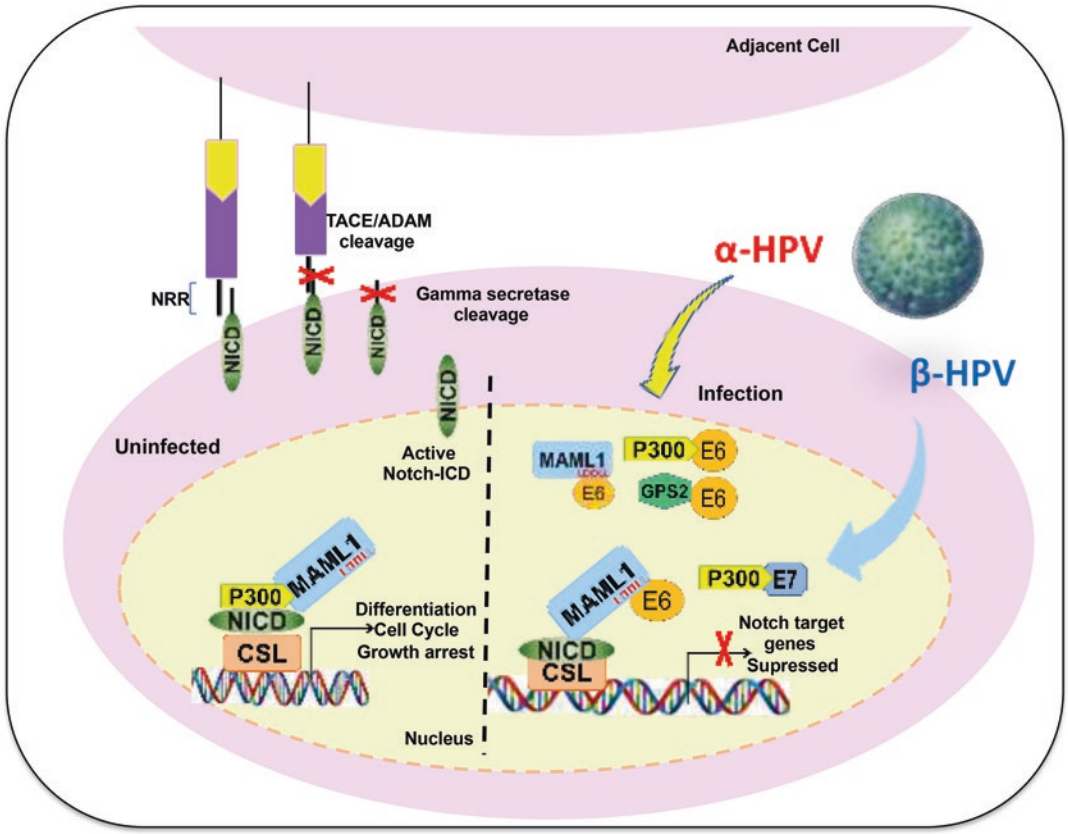

Fig. 8.4 Different mechanisms to regulate Notch signaling by HPV oncogenes in keratinocyte cells

Although β-papillomaviruses but not α-papillomaviruses have been shown to directly inhibit Notch signaling, the NOTCH pathway can also be disrupted in high-risk HPV cancers, as evidenced by Talora et al., demonstrating that cervical cancers expressing high-risk HPV subtypes downregulated NOTCH1 expression (Talora et al. 2002). High-risk α-papillomaviruses may inhibit NOTCH signaling indirectly through degradation of the TP53 tumor suppressor that, when upregulated, induces NOTCH expression (Dotto 2009). Furthermore, HPV16 E6 inhibited NOTCH cleavage and decreases NOTCH transcription which depended on TP53 degradation. Conversely, increasing NOTCH1 activity may downmodulate E6 expression via inhibition of the AP1 transcription factor complex (Wang et al. 2007). Similarly, Kranjec et al. demonstrated that the expression of HPV16 E6 disrupted NOTCH expression, which was dependent on p53 (Kranjec et al. 2017). Finally, high-risk HPVs may also target other p53 family members such as TAp63β and indirectly inhibit NOTCH1 expression (Ben Khalifa et al. 2011). Thus, both oncogenic and non-oncogenic HPV subtypes have evolved mechanisms to inhibit NOTCH signaling.

HNSCCs Display Both Inactivating and Activating NOTCH1 Mutations

The role of Notch signaling during tumor development is likely context dependent, as Notch has been shown to promote to tumorigenesis in some models while suppressing tumorigenesis in other models. NOTCH was initially described as an oncogene in hematopoietic cancers but has been shown to function primarily as a tumor suppressor in epithelial cancers. In T-cell acute lymphoblastic leukemia (T-cell ALL), activating NOTCH mutations promoted proliferation via activation of the Myc pathway (Sanchez-Martin and Ferrando 2017). By contrast, many HSNCC displayed nonsense mutations in NOTCH, resulting in truncated proteins lacking a portion of the C-terminal intracellular domains which inactivate Notch by making it incapable of transcribing downstream genes (Agrawal et al. 2011). In mouse models, loss of Notch1 drove cutaneous and oral carcinogenesis (Nicolas et al. 2003; Nyman et al. 2018). Furthermore, Retting et al. demonstrated that NOTCH1-inactivating mutations were more likely in HPV-negative cancers than in HPV-positive cancers (Rettig et al. 2015). By contrast, Izumchenko et al. demonstrated that activating NOTCH mutations, similar to those found in T-cell ALL, were also present in premalignant lesions of oral cavity cancers (Izumchenko et al. 2015). Similarly, we observed that in HPV tumor models the constitutively active Nicd1 and loss of Notch1 promoted oral tumorigenesis via distinct mechanisms. Thus, several lines of evidence define a bifunctional role for the NOTCH pathway in cancers that is likely dependent on the mutational and tissue context.

NOTCH as Tumor Suppressor in HNSCC

In head and neck cancers, loss-of-function mutations in Notch family members are among the most recurrent mutations in HNSCC (Table 8.2). The mutations identified include missense mutations, splice site mutations, and nonsense mutations that result in truncated proteins lacking the C-terminal trans-activating domain (Cancer Genome Atlas 2015). These aberrations occur predominantly in NOTCH1, but are also found in NOTCH2 and NOTCH3 (Pickering et al. 2013). Genetic alterations have also been detected that lead to reduced Notch activity by altering other Notch pathway cofactors, such as mastermind-like 1 (MAML1) (Arruga et al. 2018). In the mouse, conditional deletion of Notch signaling in epithelial progenitor cells through the expression of a dominant negative form of the Notch coactivator Maml1 promoted the expansion of preneoplastic clones harboring inactivating Tp53 mutations. Thus, loss of Notch signaling may even be an early event during head and neck carcinogenesis (Natsuizaka et al. 2017).

In vitro and in vivo, Notch1 has been shown to negatively regulate keratinocyte proliferation and initiate the process of epithelial differentiation

Table 8.2 Summary of NOTCH role as tumor suppressor in HNSCC

References	Tumor sample (*n*)	Sample	Method	Observed effect	Prediction/ Implication
Agrawal et al. (2011)	120	HNSCC tissues	Exome Sequencing	Mutated NOTCH1	Inactive protein
Stransky et al. (2011)	74	HNSCC tissues	Exome Sequencing	Mutated NOTCH1 NOTCH2 NOTCH3	Inactive protein
Pickering et al. (2013)	44	HNSCC tissues	Integrated genome analysis	Mutated NOTCH1, NOTCH2	Inactive protein
Kandoth et al. (2013)	306	HNSCC tissues	Exome sequencing-TGCA	Mutated NOTCH1	Inactive protein
Song et al. (2014)	13	HNSCC cell lines	Single molecule DNA sequencing	Mutated NOTCH	Inactive protein
Fukusumi et al. (2018)	520	HNSCC tissues	TCGA	Mutated NOTCH	Inactive protein
Pickering et al. (2013)	In vitro		Overexpression of NICD	Inhibition of tumor growth	Tumor suppressor

(Blanpain et al. 2006; Rangarajan et al. 2001). In mice, with conditional deletion of Notch1 in the skin, they developed epidermal hyperplasia leading the basal cell line carcinomas with increased Gli2 expression consistent with activation of the beta-catenin pathway (Nicolas et al. 2003). Loss of the Notch signaling pathway in adjacent non-epithelial tissues may also promote epithelial tumorigenesis. To this end, Hu et al. demonstrated that mice with conditional deletion in Notch1 co-factor Rbp-Jκ in the dermis induced keratosis followed by squamous cell carcinoma formation in the epidermis of mice. In other words, with the loss of Notch signaling in the stroma facilitated the development of pre-malignant epithelial lesions (Hu et al. 2012). Stromal cells with Notch1 loss promoted epithelial carcinogenesis by expressing higher levels of growth factors, cytokines, and matrix-metalloproteinases that promoted the proliferation and invasion of adjacent epithelial cells. Similarly, Demehri et al. demonstrated that Notch1-expressing keratinocytes can also form carcinomas when adjacent Notch1-deficient keratinocytes primed a wound-like microenvironment to promote tumor growth (Demehri et al. 2009). In chemical carcinogenesis models, mice with Notch1-deficient epithelial tissues developed dramatically more benign papillomas and squamous cell carcinomas. Namely, wild-type mice treated with 7,12-Dimethylbenz[a] anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA) developed cutaneous papillomas with frequent missense and nonsense mutations in Notch1 (Nicolas et al. 2003; Rizzo et al. 2015). Confirming NOTCH1 as a tumor suppressor in HNSCCs, Pickering et al. overexpressed NOTCH1 in various head and neck cancer cell lines, which inhibited proliferation and induced senescence (Pickering et al. 2013). Consistent with these preclinical observations, a clinical trial studying γ-secretase inhibitor in Alzheimer's disease reported an unexpected increase in nonmelanoma skin cancers further supporting the role of the Notch pathway as a tumor suppressor in epithelial cancers (Extance 2010). Thus, genetic, functional, and clinical observations support a tumor suppressive role for the Notch pathway in viral and nonviral epithelial cancers.

The NOTCH pathway may suppress tumor growth is through inhibition of the beta-catenin pathway, ΔNp63 and/or the cell cycle. Nicolas et al. demonstrated that Notch1 inactivation in the epidermis restored beta-catenin signaling in cells differentiating epithelium and keratinocytes (Fuchs and Raghavan 2002). Enhanced beta-catenin signaling was reversed by expressing a dominant active form of the Notch1 receptor, which was associated with a reduction in the signaling-competent pool of beta-catenin. In the epidermis, ΔNp63 expression, which enforces

the stem cell phenotype, is often inversely correlated with Notch activity and may inhibit the pro-differentiation effect of Notch signaling. Conversely, Notch has been shown to inhibit the expression of ΔNp63 in epidermal progenitor cells as they differentiate. In HNSCCs and other squamous cancers, ΔNp63 expression is upregulated and, consequently, a potentially important mechanism to inhibit NOTCH signaling in cancers without non-mutated NOTCH family members. Finally, Notch may function as a tumor suppressor by inhibiting the cell cycle. The NOTCH pathway has been shown to induce p21WAF1/Cip1, which disrupts cell cycle progression (Roy et al. 2007). Thus, loss of NOTCH may inhibit differentiation and promote cell cycle progression.

Conversely, activation of the NOTCH pathway has also been shown to inhibit proliferation of head and neck cancer models. Overexpression of the active NICD in oral squamous cell cancer line Tca8113 inhibited cell proliferation in vitro and in vivo accompanied by G0/G1 cell cycle arrest and apoptosis (Duan et al. 2006). In laryngeal cancer cell line Hep-2, overexpression of NOTCH1 also inhibited proliferation, causing cell cycle arrest in the G0/G1 phase and inducing apoptosis (Jiao et al. 2009). Finally, Pickering et al. demonstrated that expression of cleaved NICD or full-length NOTCH1 in HNSCC cell lines inhibited in vitro proliferation and tumorigenic growth in mice (Pickering et al. 2013).

In addition to NOTCH1, other NOTCH family members also inhibited virally induced head and neck cancers and other cancers. NOTCH3 overexpression in EBV-driven nasopharyngeal carcinoma cells inhibited cell proliferation and induced apoptosis. These tumor suppressor properties were associated with the downregulation of cell cycle and anti-apoptotic genes including CCND1, C-MYC, NFKB1, BCL2, BCL-XL, and SURVIVIN. Furthermore, xenograft spheroid formation was remarkably decreased by inhibiting NICD3, the constitutively active form of NOTCH3 (Man et al. 2012). Similarly, Lobry et al. demonstrated that loss of NOTCH signaling through the conditional deletion of Nicastrin (NCSTN), an essential component of the γ-secretase complex, or compound deletion of NOTCH1 and NOTCH2, resulted in chronic myelomonocytic leukemia. Furthermore, sequencing of Notch pathway genes revealed that ~12% of CMML patients harbored inactivating mutations in NOTCH2, NCSTN, and MAML1. Overall, these studies implicate that Notch cofactors and pathways prevented uncontrolled proliferation and transformation of myeloid cells during hematopoietic development (Lobry et al. 2011). Thus, several NOTCH family members likely have tumor suppressive functions.

NOTCH as an Oncogene in HNSCC

Initially, NOTCH was identified as an oncogene due to the identification of rare chromosomal translocations involving the NOTCH1 locus in T-cell ALLs. Namely, the NOTCH1 locus was disrupted by the t(7; 9)(q34; q34.3) chromosomal translocation placing the C-terminal region of NOTCH1 next to the TCRβ locus and allowing for constitutive expression of an activated NOTCH gene product (Yoshida et al. 2017; Sakamoto 2016). Of note, more than 50% of T-cell ALLs display this activating truncation of NOTCH1 (Weng et al. 2004; Ellisen et al. 1991).

Activation of the NOTCH pathway may also promote the growth of epithelial cancers (Table 8.3). As previously mentioned, Izumchenko et al. sequenced 95 oral cavity cancers and identified NOTCH1 mutations in 54% of invasive and 60% of preinvasive lesions (Izumchenko et al. 2015). Furthermore, oral cavity cancers from Chinese patients were frequently mutated in the HD domain, transactivation domain, and PEST domain, which are locations where the majority of activating mutations in T-cell ALL reside. Constitutive activation of the Notch signaling initiated by the direct interaction between JAG1 and NOTCH1 in HNSCC cell lines also resulting in cells with increased migration and metastatic phenotypes (Egloff and Grandis 2012; Lin et al. 2010). Abnormal expression of JAG1 triggered Notch1 activation in the HNSCC cell lines (Lin et al. 2010; Tohda and Nara 2001) as well as in adjacent endothelial

Table 8.3 Summary of NOTCH role as oncogene in HNSCC

References	Experiment	Findings and Implications
Zeng et al. (2005)	Immunohistochemistry of HNSCC tissue microarray	JAG1 was overexpressed indicating that NOTCH1 activity in HNSCCs promotes angiogenesis
Gu et al. (2010)	HNSCC cell line in collagen matrix	NOTCH1 protein positively associates with cisplatin resistance
Sun et al. (2014)	HNSCC cell lines transduced with siRNA against NOTCH1 and HEY1	Inhibition of NOTCH1 inhibited proliferation indicating NOTCH1 important for tumor growth

cells to promote angiogenesis (Zeng et al. 2005). In addition, Lin and others have shown that HNSCCs overexpressing JAG1 or NOTCH1 displayed accelerated tumor growth and angiogenesis in vivo (Zeng et al. 2005; Joo et al. 2009). In a TCGA analysis, HNSCC significantly upregulated HEY1 compared with normal tissues. Furthermore, the expression of NOTCH pathway members NOTCH1, NICD, JAG1, and HES1 was upregulated during the progression of normal tissues to dysplasia and malignancy. Immunohistochemical examination of oral tongue cancers showed increased staining of Notch1 and Notch3 protein in malignant cells compared to adjacent normal tissues. Furthermore, a positive correlation between JAG2 and NOTCH3 were found in tongue carcinoma (Zhang et al. 2011, 2013). The transcriptional alterations of NOTCH signaling pathways genes in HNSCC tumors revealed that 11 genes, including JAG1, JAG2, NOTCH3, NCSTN, DTX3L, ADAM17, DVL3, HES1, HDAC2, NCOR2, and NUMBL, were significantly upregulated, and 4, including KAT2B, MAML3, DTX1, and MFNG, downregulated (Sun et al. 2014). Finally, mutations in FBXW7, a member of the SCF ubiquitin ligase complex which regulates NOTCH1 by targeting it for proteasomal degradation, may result in increased NOTCH (O'Neil et al. 2007). Thus, HNSCCs demonstrate genetic and transcriptional evidence for the activation of the NOTCH pathway as an oncogenic mediator of tumor growth.

The first proof confirming increased NOTCH signaling promotes solid tumor development was observed by integration of the mouse mammary tumor virus into the Notch4 gene. This integration resulted in mammary tumorigenesis via LTR-driven expression of the truncated, constitutively active form of the Notch4 gene (Uyttendaele et al. 1996). Similarly, we observed that increasing the expression of NICD1 in primary HPV oral tumors promoted tumor growth via upregulation of gene expression pathways encompassing MYC and other genes that promote cell proliferation. Furthermore, compared to other invasive SCCs, NOTCH1 was overexpressed in verrucous carcinomas, a rare variant of oral cancer with pushing boarders rather than deeply invasive (Zhong et al. 2015). NOTCH1 is also significantly related to cervical lymph node metastasis in oral tongue cancers (Joo et al. 2009; Zhang et al. 2011). Similarly, Leethanakul et al. demonstrated that NOTCH2 expression was associated with lymph node metastasis in HNSCCs (Leethanakul et al. 2000). Finally, the NOTCH pathway may play an important role in cell renewal and survival as upregulation of NOTCH1-mediated chemoresistance by promoting self-renewal and stemness in HNSCC cell lines (Zhao et al. 2016).

Activation of the NOTCH pathway may also promote the growth of non-squamous head and neck cancers. NOTCH1 is the most frequently altered gene in adenoid cystic carcinoma (ACC), a relatively rare tumor of the head and neck. In this disease, NOTCH1 mutations were associated with higher NICD expression and poor prognosis. Furthermore, NOTCH1 may be a targetable gene in ACCs as brontictuzumab, a monoclonal antibody to NOTCH1, inhibited NOTCH1-mediated signaling both in patients and xenograft models and was associated with clinical efficacy in patients (Ferrarotto et al. 2018). Similarly, some patients with ACC also responded to γ-secretase inhibitors, which inhibit the cleavage NOTCH1 and activation of the NOTCH pathway (Massard et al. 2018). Finally, the Notch pathway

has been shown to promote the growth of other solid tumors including breast cancer, NSCLCs, colorectal cancer, pancreatic cancer, and medulloblastoma (Suman et al. 2014; Brzozowa-Zasada et al. 2017; Du et al. 2018; Kumar et al. 2019). Overall, it confirms that NOTCH is playing an oncogenic role in many epithelial cancers including some HNSCCs.

Activation of the NOTCH pathway may promote head and neck tumor growth via activation of pathways involved in cell proliferation and anti-apoptosis. NOTCH1 has been shown to directly induce c-MYC expression in T-cell acute lymphoblastic leukemia (Weng et al. 2006; Herranz et al. 2014). NOTCH2 also affects cell growth and apoptosis, and knockdown of NOTCH2 inhibited the migration and invasion abilities and decreased the expression levels of its downstream genes such as c-MYC and BCL-2 (Zou et al. 2016). In addition, NOTCH activation increased FGF1 gene expression and cell invasion in oral squamous cell carcinomas (Weaver et al. 2016). Furthermore, inhibition of the NOTCH pathway is associated with decreased phosphorylation of AKT, a serine/threonine-specific protein kinase involved in metabolism, cell proliferation, and migration (Das et al. 2016). In the TCGA data set, cancers with wild-type NOTCH1 exhibited increased expression of the NOTCH1 target genes HEY1 and HES1 as well as an associate with increased BCL-2 expression (Fukusumi and Califano 2018).

The Mutational Context May Determine NOTCH's Role as an Oncogene or Tumor Suppressor

The deciding factors that determine whether NOTCH acts as a tumor suppressor or oncogene remains unresolved. It is unlikely that cell type of origin is the primary factor, given that NOTCH pathway activation or loss is present in cancers of the same tissue type and, in cell and animal models, activation or loss of the NOTCH pathway promotes tumor growth. Rather, it is likely that the mutational context and the timing during which mutations arise help to determine whether the activation or loss of NOTCH pathway promotes tumor growth. As we have described, NOTCH activates pro-tumorigenic pathways including activation of AKT and c-MYC pathways as well as tumor suppressive signals comprising the commitment of differentiation programs and the inhibition of ΔNp63 and the beta-catenin pathway. During carcinogenesis, cells that acquire mutations that block differentiation program and bypass cell cycle arrest may benefit from NOTCH activation which further stimulates proliferation pathways. To this end, we observed that in HPV oral tumors, where E7 can partially block squamous differentiation, NOTCH can function as a tumor suppressor. In this circumstance, loss of NOTCH may also promote tumor growth by helping to enforce a stem cell phenotype and promote the expression of genes involved in cell migration (Zhong et al. 2015). Conversely, in early premalignant lesions, loss of NOTCH may be necessary for transformation. Thus, the role of NOTCH as a tumor suppressor or oncogene likely depends on the time and context of other mutations during carcinogenesis.

Conclusions

Notch pathway is critical in HSNCCs as 66% of cancers carry some sort of genetic alteration in either of the NOTCH 1–4 signaling proteins (Agrawal et al. 2011). NOTCH has been reported to have both oncogenic and tumor suppressive roles in cancer, which are likely dependent on the cellular context. A variety of both activating and inactivating NOTCH mutations have now been observed in various HNSCC patients. NOTCH activation has been demonstrated in multiple cancers including T-cell ALL, pancreatic cancer, breast cancer, prostate cancer, liver cancer, cervical cancer, and HNSCC among others (Sun et al. 2014). By contrast, NOTCH inactivation is also frequently observed in many epithelial cancers including HNSCCs. The complexity of the opposing roles for the NOTCH pathway in HNSCC and other cancers makes it difficult to implement novel therapeutic approaches. Rather, one must look at the NOTCH mutations specific

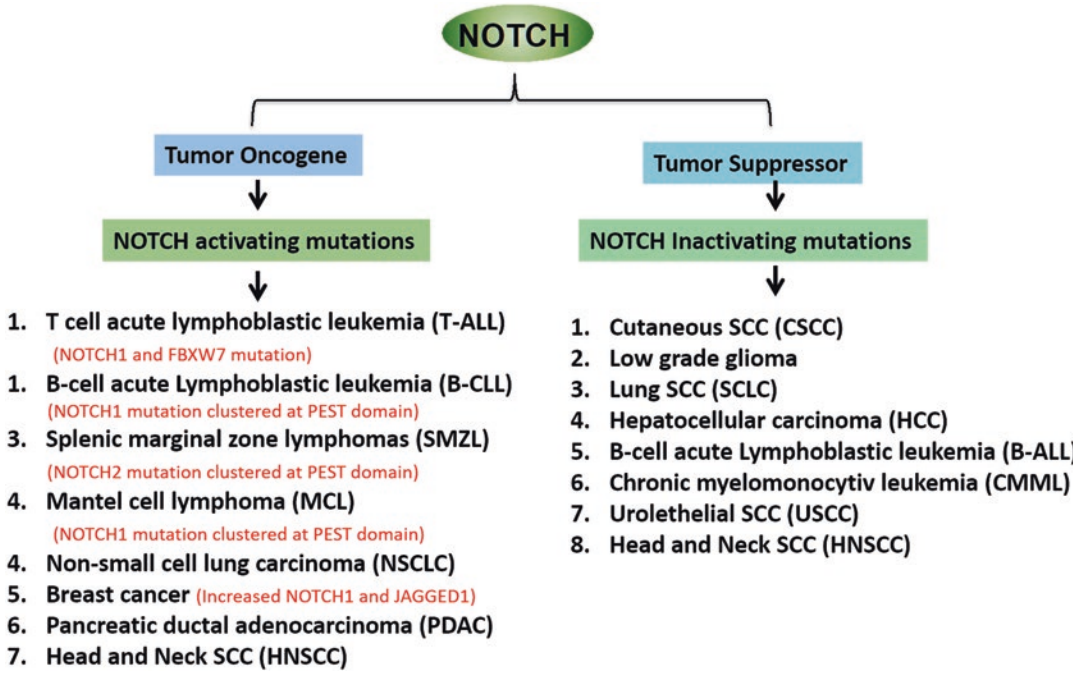

Fig. 8.5 Dual role of Notch as oncogene and tumor suppressor in various cancers including HNSCCs

to individual cancers as well as the context of other mutated and/or altered gene in order to rationally target the NOTCH pathway. Unraveling the molecular decisions that determine the oncogenic or tumor suppressive role of NOTCH will unravel new strategies for a targeted therapy for HPV-associated and HPV-negative HNSCC (Fig. 8.5).

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9 The Impact of Notch Signaling for Carcinogenesis and Progression of Nonmelanoma Skin Cancer: Lessons Learned from Cancer Stem Cells, Tumor Angiogenesis, and Beyond

Jörg Reichrath and Sandra Reichrath

Abstract

Since many decades, nonmelanoma skin cancer (NMSCs) is the most common malignancy worldwide. Basal cell carcinomas (BCC) and squamous cell carcinomas (SCC) are the major types of NMSCs, representing approximately 70% and 25% of these neoplasias, respectively. Because of their continuously rising incidence rates, NMSCs represent a constantly increasing global challenge for healthcare, although they are in most cases nonlethal and curable (e.g., by surgery). While at present, carcinogenesis of NMSC is still not fully understood, the relevance of genetic and molecular alterations in several pathways, including evolutionary highly conserved Notch signaling, has now been shown convincingly. The Notch pathway, which was first developed during evolution in metazoans and that was first discovered in fruit flies (*Drosophila melanogaster*), governs cell fate decisions and many other fundamental processes that are of high relevance not only for embryonic development, but also for initiation, promotion, and progression of cancer. Choosing NMSC as a model, we give in this review a brief overview on the interaction of Notch signaling with important oncogenic and tumor suppressor pathways and on its role for several hallmarks of carcinogenesis and cancer progression, including the regulation of cancer stem cells, tumor angiogenesis, and senescence.

Keywords

Angiogenesis · Cancer · Cancer stem cells · Cancer treatment · Notch · Nonmelanoma skin cancer · Notch signaling · Notch pathway · Skin cancer · Tumor angiogenesis

Abbreviations

AE	Adverse event
AK	Actinic keratosis
Ang	Angiopoietin

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BCC	Basal cell carcinoma
BMP	Bone morphogenic protein
cKO	Conditional knockout
CSC	Cancer stem cell
CSL	CBF-1, Su (H), Lag-1-type transcription factor (also termed RBP-J)
DBD	DNA-binding domain
Dll	Delta-like
E	Embryonic day
FGF	Fibroblast growth factor
GLI1	Glioma-associated oncogene homolog 1
Hes	Hairy and enhancer of split
Hf	Hair follicle
HPV	Human papilloma virus
Hrt	Hes-related transcription factor
IP	Intermediate progenitor
IPC	Intermediate progenitor cell
JAG	Jagged
KO	Knockout
MAML	Mastermind-like
MET	Mesenchymal-to-epithelial transition
MMP	Matrix metalloproteinase
MR	Mortality rate
NID	Notch intracellular domain
NMSC	Nonmelanoma skin cancer
NO	Nitric oxide
OD	Oligomerization domain
PDGF	Platelet-derived growth factor
PDT	Photodynamic therapy
PTCH	Patched
RBP-J	Recombinant recognition sequence-binding protein at the J_κ site (also termed CSL)
SCC	Squamous cell carcinoma
Sema	Semaporin
Shh	Sonic hedgehog
SMC	Smooth muscle cell
TA	Transactivation domain
TLR	Toll-like-receptor
UVR	Ultraviolet radiation
VEGF	Vascular endothelial growth factor
Wnt	Wingless-related integration site

Ancient Friends, Revisited: A Short Introduction to the Relevance of Notch Signaling for Skin and Cancer

Although most types of nonmelanoma skin cancers (NMSCs) are not documented in the cancer registries of most countries, there is no doubt that they are since many decades the most common malignancies worldwide (Reichrath and Reichrath 2012a; Reichrath and Rass 2014). Basal cell carcinomas (BCCs) and cutaneous squamous cell carcinomas (SCCs), that are in general nonlethal and curable (e.g., by surgery), represent in many populations approximately 70% and 25% of NMSCs, respectively (Reichrath and Reichrath 2012a; Reichrath and Rass 2014). Because of their continuously rising incidence rates, NMSCs represent a constantly increasing challenge for global healthcare (Reichrath and Reichrath 2012a). BCCs and SCCs share many characteristics, including that both of them may be induced by solar or artificial ultraviolet radiation (UVR), but they are very different from etiology to progression (Reichrath and Reichrath 2012a; Reichrath and Rass 2014). Both UVA and UVB may cause DNA damage and immunosuppression, which play crucial roles in skin photocarcinogenesis (Reichrath and Reichrath 2012a; Reichrath and Rass 2014). UVB can be directly absorbed by DNA molecules, thereby resulting in characteristic UV-signature DNA damages (Reichrath and Reichrath 2012a; Reichrath and Rass 2014). UVA, on the other hand, may, to a lesser extent, also exert DNA damage, through inducing cellular reactive oxygen species (ROS) which then induces oxidative DNA damages (Reichrath and Reichrath 2012a; Reichrath and Rass 2014). Although the photocarcinogenesis of NMSC is still not fully understood, it has shown promise as a well-suited model to investigate the relevance of various signaling networks, including pathways that are also of relevance for embryonic development and for the multistep-

carcinogenesis of solid tumors (Reichrath and Reichrath 2012a; Reichrath and Rass 2014). In NMSC, the relevance of genetic and molecular alterations in many pro- and anticarcinogenic pathways, including evolutionary highly conserved Notch signaling, has now been shown convincingly (Reichrath and Reichrath 2012a). The tale that earned the gene Notch, its name started over a century ago at Olivet College (Olivet, Michigan, USA), when the American Scientist John S. Dexter observed and described the characteristic notched-wing phenotype (a nick or notch in the wingtip) in his mutant fruit flies *Drosophila melanogaster* (Dexter 1914; Reichrath and Reichrath 2020a, b, c). Notably, the Notch pathway is simple in design but has a striking versatility in function (Andersson et al. 2011; Reichrath et al. 2010; Reichrath and Reichrath 2020a, b, c). Notch signaling first developed during evolution in metazoans and was first discovered in fruit flies (*Drosophila melanogaster*) (Gazave et al. 2009; Richards and Degnan 2009). It orchestrates and governs from sponges to humans during embryonic development and in adult tissues cell fate decisions and many other fundamental cellular processes (Andersson et al. 2011; Reichrath and Reichrath 2020a, b, c). Besides the ability to activate and orchestrate cell proliferation, thereby keeping precursor and stem cells in a nondifferentiated state as well as generating and maintaining stem cells, these flagship functions include regulation of important hallmarks during the multistep process of skin carcinogenesis, including initiation, promotion, and progression of cancer cells. In general, these functions involve canonical, ligand-dependent stimulation of Notch receptors (McIntyre et al. 2020). However, ligand-independent Notch activation has also been observed in several distinct cellular contexts (rev. in McIntyre et al. 2020; Reichrath and Reichrath 2020a, b, c). During the last decades, a huge mountain of new scientific information – ranging from the elucidation of the Notch pathway (Kidd et al. 1986; Kiernan et al. 2001, 2006, 2007; reviewed by Bray 2016; Kopan and Ilagan 2009; McIntyre et al. 2020; Reichrath and Reichrath 2020a, b, c), to the generation of knockouts in model organisms and the discovery of mutated Notch genes in humans (Gridley 2003) – has confirmed an essential role for Notch signaling for various types of cancers, including NMSC. As outlined above, environmental hazards, including solar and artificial UV-radiation, represent important risk factors for carcinogenesis of both melanoma and NMSC (Reichrath and Rass 2014). Notably, the skin (with the epidermis and its adjoining structures, including hair follicles (HF) and associated sebaceous glands; that together comprise the pilosebaceous unit) is not only the human body's largest organ but also its first line of defense against UV-radiation and many other environmental hazards, providing protection from dehydration, injury, and infection (Shi et al. 2017). Hair follicles have been described as self-renewing structures that continuously generate new epithelial cells to guarantee skin integrity and to renew the skin and its epidermal appendages in response to injury or environmental hazards (Rishikaysh et al. 2014). It is well known that Notch signaling is of high importance for skin homeostasis and wound repair, that both depend on the presence of epithelial stem cells as the primary source for regenerative cells (Blanpain and Fuchs 2006; Shi et al. 2017). Multipotent stem cells that reside within the epidermis and in the bulge region of HFs can give rise to a variety of different cell types, including those forming HFs, interfollicular epidermis, and associated epithelial glands (Shi et al. 2017). Notch signaling has been shown to govern proliferation and differentiation of these cell types, two processes whose alterations have the potential to disrupt normal cell growth and skin homeostasis (Shi et al. 2017) and may finally result in malignant transformation of these cells.

Besides certain other disorders of the skin, such as chronic wounds, skin atrophy, skin fragility, hirsutism, and alopecia, NMSC show characteristic features that are well in line with a disorder of skin stem cells (Najafzadeh et al. 2015; Shi et al. 2017). In NMSC and many other malignancies, it has been hypothesized that tumor formation is caused by inappropriate stim-

ulation/regulation of distinct cellular signaling pathways (including Notch signaling), thereby activating these stem cells or their immediate pluripotent progenitors (Burkert et al. 2006; Shi et al. 2017). In line with this concept is the observation that several types of NMSCs can obviously be derived from HFs, an assumption that is supported by characteristic histological findings and the detection of specific molecular markers both in HFs and in skin malignancies (Jahoda and Reynolds 2000; Shi et al. 2017). It has been concluded that understanding the molecular mechanisms by which proliferation and differentiation are regulated in skin appendages may provide important insights into the molecular basis of NMSC and other diseases, and may also identify promising targets for treatment intervention (Shi et al. 2017). In this review, we give a brief overview on the role of Notch signaling for the multistep process of photocarcinogenesis of NMSC (including the roles of the Notch pathway for the regulation of cancer stem cells (CSCs) and tumor angiogenesis), for progression and clinical management of NMSC (including Notch's role as an emerging therapeutic target). Because understanding the fundamental role of Notch for embryonic development of skin, HFs, and other appendages is of critical importance for understanding the relevance of Notch signaling for skin carcinogenesis, we will also give a short introduction on this topic.

A Snapshot on the Role of Notch Signaling for Embryonic Development and Tissue Homeostasis of Skin and Hair Follicles

It has been convincingly shown that the evolutionary, highly conserved Notch pathway governs fundamental developmental processes that include binary decision, lateral inhibition, and boundary formation (Artavanis-Tsakonas et al. 1999; Reichrath and Reichrath 2020a, b, c). In general, Notch-mediated cell–cell communication is context and cell-type dependent and exerted by coordinated, differential expression of distinct Notch receptors and corresponding ligands on the surface of adjacent cells (reviewed in McIntyre et al. 2020). In mammals, four evolutionary, highly conserved transmembrane Notch receptors (Notch1–4) have been identified, that can be activated via five corresponding ligands of the *Delta-like* (Dll 1, 3, and 4) and *Jagged* (JAG1 and 2) families (Kiernan et al. 2001; reviewed in McIntyre et al. 2020), In general, neighboring cells stimulate each other to produce elevated levels of ligands, thereby inducing an increased activation of Notch receptors (Artavanis-Tsakonas et al. 1999; reviewed in McIntyre et al. 2020). In most cases, elevated expression of ligands with subsequent Notch activation results in cellular differentiation (and cell growth arrest), thereby regulating the cluster size of cell populations (Artavanis-Tsakonas et al. 1999; reviewed in McIntyre et al. 2020). At the molecular level, it has been shown that in the canonical Notch-signaling pathway, ligand-induced Notch receptor stimulation results in cleavage of the intracellular domain of the Notch receptor (NID) (reviewed in McIntyre et al. 2020). The NID then consecutively translocates to the nucleus where it forms a ternary complex with the transcriptional coactivator, Mastermind-like (MAML) protein, as well as the DNA-binding protein, CBF-1, Su (H), Lag-1-type transcription factor (CSL, also termed Recombinant recognition sequence binding protein at the J_{κ} site, RBP-J), which have been shown to direct specific binding to response elements in DNA regions of target genes and to regulate target gene expression (reviewed in McIntyre et al. 2020). Until today, only a limited number of Notch target genes have been identified and characterized, most importantly basic-helix–loop-helix proteins of the hairy and enhancer of split (*Hes*) and *Hes*-related transcription factor (*Hrt*) families, which function as transcriptional repressors (reviewed in McIntyre et al. 2020).

Notch Signaling in Skin: Simple in Design but Versatile in Function

Recent scientific findings indicate that Notch signaling orchestrates the process of epidermal differentiation and proliferation through the

sequential activation of different Notch ligands, receptors, and downstream pathways. Notch receptors and corresponding ligands are present in the skin (Reichrath and Reichrath 2012a, b), although until today, most of their particular functions are still uncertain. It has been shown that Notch receptors and ligands are differentially expressed in the different cell layers of the viable epidermis (reviewed in Reichrath and Reichrath 2012a). In healthy skin Notch receptor 1 and its corresponding ligands, Dll1 and JAG1 are present in all cell layers of the viable epidermis, with pronounced expression of Dll1 and JAG 1 in the epidermal basal layer (Table 9.1). It has been observed in various cell types that Delta/Notch signaling is increased in cells that undergo a normal differentiation program, as in human keratinocytes of cell layers of the normal adult epidermis. In contrast, activity of Notch signaling has been described to be decreased in psoriasis vulgaris and other hyperproliferating skin diseases. In line with these investigations, it was reported that loss of Notch receptor1 in young mice induces hyperproliferation of the basal epidermal layer and deregulates expression of multiple differentiation markers, including reduced expression of p21 and elevated expression of Gli2. In epidermal keratinocytes, activation of Notch receptor 1 has been shown to induce p21 expression in a CBF-1, Su (H), Lag-1 (CSL)-type (also termed RBP-J) transcription factor–dependent manner, resulting in cell cycle withdrawal and terminal differentiation. In addition, stimulation of Notch receptor 1 directly promotes caspase 3 activity, that is required for terminal differentiation of embryonic keratinocytes.

The importance of Notch signaling for skin embryogenesis is underlined by characteristic cutaneous findings in several inherited syndromes, including Alagille syndrome (Kamath et al. 2004, 2012, 2013; McCright et al. 2001, 2002, 2006) and Adams–Oliver syndrome. Adams–Oliver syndrome is a rare genetic disorder that has been linked to mutations in several different genes, including DLL4 (OMIM 605185; cytogenetic location: 15q15.1) and NOTCH1 (OMIM 190198; cytogenetic location: 9q34.3), as well as in RBP-J (OMIM 147183; cytogenetic location: 4q15.2), EOGT (OMIM 614789; cytogenetic location: 3p14.1), ARHGAP31 (OMIM 610911; cytogenetic location: 3q13.2–3q13.33), and DOCK6 (OMIM 614194; cytogenetic location: 19p13.2) (reviewed in Mašek and Andersson 2017; Meester et al. 2019; Reichrath and Reichrath 2020a). Adams–Oliver syndrome is diagnosed based on the presence of aplasia cutis congenita and several other clinical hallmarks, namely terminal transverse limb malformations and a partial absence of skull bones (reviewed in Mašek and Andersson 2017; Meester et al. 2019; Reichrath and Reichrath 2020a; Zanotti and Canalis 2016). Typically, aplasia cutis congenita is found in the skull region, however other body parts, including the abdomen, may also be affected (reviewed in Meester et al. 2019; Zanotti and Canalis 2016). The severity and symptoms of aplasia cutis congenita may greatly vary (reviewed in Meester et al. 2019; Zanotti and Canalis 2016). At birth, the affected skin region typically presents as healed but scarred skin, and skin histology shows characteristic findings that may include absent epidermis, dermal atrophy, and a lack of elastic fibers and other skin structures (reviewed in Meester et al. 2019; Reichrath and Reichrath 2020a; Zanotti and Canalis 2016). However, symptoms may range from a localized region with complete absence of skin to patches of skin that lack hair (reviewed in Meester et al. 2019; Zanotti and Canalis 2016).

A large body of convincing evidence from clinical and laboratory investigations has shown the importance of Notch signaling for the embryonic development of all anatomical structures of the skin, including the epidermal compartment, HFs, and other appendages. It was demonstrated that in response to external cues, embryonic skin cells have to make a cell fate decision whether or not to differentiate and generate stratified epidermis, or to invaginate and initiate morphogenesis of HF (Fuchs 2007). It has been demonstrated that the cell fate decisions of epidermal keratinocytes whether or not to transit from basal to suprabasal epidermal cell layers begin around embryonic day 13.5 (E13.5). At this time, the activation of Notch receptors by

Table 9.1 Notch signaling in skin embryology and in nonmelanoma skin cancer: lessons learned from in vitro investigations and animal studies

Topic		Clinical and laboratory findings	References (Selection)
Relevance of Notch signaling for NMSC	Pathology of NMSC: HFs and BCCs	*Pathology of HFs and BCCs: examples for "ordered" and "disordered" variants of skin appendage growths, respectively*	Dahmane et al. (1997)
		A significant subset of BCCs may be directly HF-derived	Garber (2007)
		Cells of both BCCs and HFs have the ability to indefinitely and repeatedly proliferate, a key mechanism that is responsible for maintaining a tumor mass or regular hair fiber production	Grachtchouk et al. (2000)
		The stem cells of the HF bulge region and adjacent cells represent a potential primary source of BCCs derived from HFs	Hutchin et al. (2005)
		All BCCs, HF-derived or not, may express similar fundamental growth mechanisms to those that regulate HF growth and cycling	Jayaraman et al. (2014)
		The primary mechanism by which most BCCs develop, a constitutive activation of the Hedgehog pathway, is a fundamental regulatory mechanism in HF development	Liu et al. (2008)
		Notch and other pathways involved in pathogenesis of BCCs	Lowell et al. (2000)
		Mutations in genes encoding for components of the sonic hedgehog (Shh) and patched (PTCH) signaling pathways are the molecular hallmarks for pathogenesis of BCCs; significance of mutations in *NOTCH*1 and *NOTCH*2 was also shown	McMahon et al. (2003)
		Shh signaling is required for the proliferation and normal cycling of HF epithelium. Modifications of Shh signaling can result in tumor development in tissues of different origins Hyperactivation of the Shh signaling pathway is found in several HF-derived tumors and in BCCs. Enhanced expression of GLI1 and GLI2 (indicating increased Shh signaling) was demonstrated in BCCs and in HF root sheaths, compared with normal skin	Nicolas et al. (2003)
		Several signaling pathways involved in regulation of stem cell functions (such as the process of cellular self-renewal), including Notch, sonic hedgehog (Shh) and Wingless-related integration site (Wnt) signaling, are active in normal HFs and in BCCs, although their roles for BCC growth remain until today only poorly understood	Oro et al. (1997)
		Defining gene expression patterns and pathways in BCCs that are distinct from HF growth and cycling may lead to a better understanding of the abnormal proliferation that these cells undergo in the development of NMSC	Yamamoto et al. (2003)

(continued)

Table 9.1 (continued)

Topic		Clinical and laboratory findings	References (Selection)
		Notch signaling pathway activity is suppressed in BCCs as compared to HFs	Purow (2012)
		Several key Notch signaling factors showed significant differential expression in BCCs compared with HFs indicating that selected Notch pathway genes are differentially activated and inhibited in BCCs, which may be due to positive feedback, and reciprocal negative feedback, from differences in Delta and Notch cell surface expression, or the irregular activation of downstream Notch signaling pathway genes	Rishikaysh et al. (2014)
		Downstream components of the Notch pathway, including the transcription factor RBP-J and downstream target genes of the Hes and Deltex families, show a high expression in hair shafts compared with BCCs and normal skin	Shi et al. (2017)
		By contrast, two genes that affect the co-repression of RBP-J, CTBP1 and CREBBP, show significantly lower expression in HFs compared with BCCs. Studies suggest a reduced expression of downstream genes of the Notch/RBP-J signaling pathway in BCCs. Deletion of RBP-J from follicular stem cells results in an aberrant cell fate switch that leads to the establishment of epidermal progenitors and basal cells the Notch/RBP-J signaling pathway is strongly activated in HFs. Since the Notch signaling pathway promotes a stem cell phenotype in skin the degree of Notch signaling pathway activation may be important for HF stem cell proliferation and differentiation	Thelu et al. (2002)
		High level of Notch/RBP-J signaling may be required for the formation and maintenance of HF	Weng and Aster (2004)
		Loss of RBP-J action in BCCs may promote cells toward a more stem, or progenitor, cell-like status, enabling basal cell tumor growth	Wuest et al. (2007)
		Notch signaling as a target for BCC treatment	Yamamoto et al. (2003)
		Notch signaling via NID translocation into the nucleus, and subsequent binding to the transcription factor RBP-J, represents an important stage of BCC development, identifying RBP-J signaling as a promising target for development of new BCC therapies	
		The role of Notch signaling as promising therapeutic target for NMSCs is supported by demonstrating that JAG1 protein is upregulated in BCCs following topical treatment with imiquimod (a small synthetic compound that stimulates Toll-like-receptor 7 (TLR-7), which is present in plasmacytoid dendritic cells, macrophages, and monocytes, thereby causing an increased production of inflammatory cytokines, including interferon-α and leading to potent stimulation of antitumor immunity that finally results in tumor destruction)	

(continued)

Table 9.1 (continued)

Topic		Clinical and laboratory findings	References (Selection)
		Topical treatment of BCCs with imiquimod mRNA (expression of Notch1, JAG1, and Dll1 transcriptionally upregulated in tumor cells of BCCs (real-time PCR; $n = 6$) Minor increase of Notch1 protein expression on infiltrating cells and strong increase in JAG1 protein expression in regressing BCCs (immunohistochemistry; $n = 6$) Conclusion: Imiquimod may act as a stimulator of the Notch pathway in sBCC tumor cells by upregulating protein expression of JAG1. Imiquimod may exert tumor suppressor function via induction of Notch signaling, which together with its proinflammatory properties may result in tumor regression	
	Pathology of NMSC: Notch and SCCs	*Notch signaling and carcinogenesis of cutaneous SCCs (cSCCs)*	Dahmane et al. (1997) Garber (2007)
		Crosstalk between Notch and p53 signaling	Grachtchouk et al. (2000) Hutchin et al. (2005)
		Mutations in the tumor suppressor p53 (TP53) are a hallmark of photocarcinogenesis of cSCCs	Jayaraman et al. (2014) Koch and Radtke (2007)
		Cross-regulations among Notch signaling and p53 family members, p63 (TP63), and p73 (TP73) contributes to the photocarcinogenesis of cutaneous SCCs	Kouwenhoven et al. (2010) Lang et al. (2004)
		p53 exerts its tumor suppressive function by inducing expression of differentiation-stimulating Notch1 and the cell cycle inhibitor p21/CDKN1A, among other target genes	Lane and Levine (2010) Liu et al. (2008)
		A complex crosstalk exists between p63 and the Notch signaling: p63 directly induces JAG1 and Notch expression, as well as the Notch target IRF6, promoting initial steps of terminal differentiation. At the same time, p63 suppresses expression of Notch downstream target genes, p21/CDKN1A and Hes1, sustaining cell cycle progression and repressing late stages of differentiation. Notch and IRF6 counteract p63 activity in a negative feedback loop	Lokshin et al. (2007) Lowell et al. (2000)
		Little is known about impact of p73 for photocarcinogenesis of cutaneous SCCs. In other cell types p73 positively regulates JAG1 and JAG2	McMahon et al. (2003) Missero and Antonini (2014)
		The effects of Notch1 deletion on multistage skin carcinogenesis	Nicolas et al. (2003) Oro et al. (1997)
		Notch1 deletion in epidermal keratinocytes causes skin carcinogenesis, while in contrast Notch1 acts in most other tissues as a proto-oncogene	Purow (2012) Rishikaysh et al. (2014)

(continued)

Table 9.1 (continued)

Topic		Clinical and laboratory findings	References (Selection)
		Deleting Notch1 either before or after DMBA treatment in the *K14CreERT* system indicates that loss of Notch1 is not involved in the initiating event of multistage skin carcinogenesis	Roemer (2012) Shi et al. (2017)
		Notch1 loss acts as a skin cancer-promoting event. Delaying Notch1 deletion in *K14CreERT* mice until after the tumor-promotion stage of carcinogenesis demonstrated that late deletion of Notch1 contributes to malignant progression of benign papillomas (a phenotype that is observed upon loss of p53 but not loss of $p21^{WAF1/Cip1}$, a specific Notch1 target in skin)	Thelu et al. (2002) Weng and Aster (2004)
		The main effect of Notch1 loss in skin carcinogenesis may be to provide the initiated cells with a proliferative signal to promote tumor growth and proceed to invasive skin cancer	Wuest et al. (2007) Yamamoto et al. (2003)
		It has been speculated that this proliferative signal is located downstream of Notch1 loss and could be originated from within the initiated cells, supporting Notch1's role as a classical tumor suppressor in epidermal keratinocytes	Yang et al. (2010)
		Expression of Notch1 and its ligands in normal skin and in NMSC	Dahmane et al. (1997) Erb et al. (2008)
		Varies in the different layers of the epidermis (most studies report immunoreactivity for Notch1 and its corresponding ligands Dll1 and JAG1 in all cell layers of the viable epidermis, with pronounced expression of the latter two in the basal layer	Garber (2007) Grachtchouk et al. (2000)
		In BCCs, the protein expression of Notch receptors and corresponding ligands, Dll1 and JAG1 is markedly lowered in tumor regions as compared to healthy epidermis	Hutchin et al. (2005) Jayaraman et al. (2014)
		One immunohistochemical study reports absent immunoreactivity for Notch1 in normal epidermis and in BCCs, while Notch2 and its downstream target gene Hes1 were detected in both cytoplasm and nuclei of normal skin epithelia (8/8) and, of BCCs (with reduced detection rates)	Kouwenhoven et al. (2010) Lane and Levine (2010)
		Protein expression of Notch1 and its corresponding ligands Dll1 and JAG1 was reduced/undetectable in BCC regions comprised of palisading cells penetrating the dermis	Lang et al. (2004) Liu et al. (2008)
		Ablation of Notch1 from epidermal cells in mice leads to an uncontrolled proliferation of the basal epidermal layer and finally results in BCC-like tumors	Lokshin et al. (2007) Lowell et al. (2000)
		In BCCs, in absence of Notch1, Dll1, and JAG1, missing or reduced Notch signaling activity may cause disordered epidermal terminal differentiation and proliferation	McMahon et al. (2003) Missero and Antonini (2014)

(continued)

Table 9.1 (continued)

Topic		Clinical and laboratory findings	References (Selection)
		BCCs lack expression of Fas, but strongly express Fas ligand, which may help prevent attack from surrounding immune-effector cells, while also lacking Fas, potentially to make the tumor cells resistant to apoptosis. Fas ligand is a type II transmembrane protein that can induce apoptosis upon binding to Fas. Decreasing Fas expression in tumor cells and simultaneously upregulating the expression of Fas ligand to induce apoptosis in Fas-expressing T-cells is a mechanism by which some malignant tumors resist Fas ligand-mediated T-cell cytotoxicity	Nicolas et al. (2003) Oro et al. (1997)
		Stimulating Notch signaling with JAG1 (by adding exogenous JAG1 into BCC cell culture) induces apoptosis of BCC cells by increasing Fas ligand mRNA and protein expression and downstream caspase-8 activation	Purow (2012) Rishikaysh et al. (2014)
		In animal models, mice lacking Notch1 spontaneously develop epidermal skin tumors that display basal cell carcinoma (BCC)-like phenotype	Shi et al. (2017) Thelu et al. (2002)
		In absence of Notch1, Dll1, and JAG1, missing or decreased Notch signaling may lead to disorder in epidermal differentiation and proliferation, and promotes formation of BCCs	Wang et al. (2012) Weng and Aster (2004)
		Impaired Notch signaling is also reported to promote the development of cutaneous squamous cell carcinoma (SCC), as outlined above, and malignant melanoma (MM). In summary, it can be assumed that in contrast to other tissues, Notch seems to function in the skin as a tumor suppressor	Wuest et al. (2007) Yamamoto et al. (2003)
		Notch receptor family members 1 and 2 may exert equivalent or reverse biological effects in cell type-dependent fashions	Yang et al. (2010)
Skin embryology	Notch signaling in skin development	*Cell fate decisions of epidermal keratinocytes whether or not to transit from basal to suprabasal cell layers* Begin around E13.5 Associated with stratification of the epidermis Associated with activation of notch receptors by corresponding ligands (on the molecular level mediated by enzymatic cleavage of NID and its translocation to the nucleus, where it associates in suprabasal keratinocytes with DNA-binding protein RBP-J to regulate downstream target genes)	Blanpain et al. (2006) Fuchs (2007)
Notch and angiogenesis	Notch signaling, angiogenesis, and congenital disorders	*Congenital disorders associated with cardiovascular defects or abnormal angiogenesis and linked to mutations in the Notch pathway*	Allard et al. (2004) Benedito et al. (2009) Boucher et al. (2013)

(continued)

Table 9.1 (continued)

Topic		Clinical and laboratory findings	References (Selection)
		Alagille syndrome (mutations in *JAG1* or *NOTCH2),* characterized by congenital heart disease (especially pulmonary artery stenosis, and vascular disease including a predisposition to intracranial bleeding)	Djokovic et al. (2015) Folkman and Klagsbrun (1987), Folkman (1996) Gonzalez-Perez and Rueda (2013)
		Hajdu–Cheney syndrome (HCS) (mutations in *NOTCH2*), clinical findings may include cardio-vascular defects	Hamada et al. (1999) Hellström et al. (2007)
		Adams–Oliver syndrome (mutations in several genes, including *Dll-4, NOTCH1, RBPJ, EOGT, ARHGAP3*, and *DOCK6*), clinical findings may include congenital heart defects (around 23%), pulmonary or portal hypertension, vascular anomalies (including dilated surface blood vessels, resulting in marbled appearance of affected skin areas, termed cutis marmorata teleangiectatica) and retinal hypervascularization	High et al. (2008) Kiernan et al. (2007) Lee et al. (2016)
		Cerebral autosomal–dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (mutations in *NOTCH3*), clinical findings include arteriopathy that shows breakdown of vSMC that causes multiple ischemic strokes	Lobov et al. (2007) Mašek and Andersson (2017) McCright et al. (2006)
		Early onset arteriopathy with cavitating leukodystrophy (mutations in *NOTCH3*), clinical findings include childhood-onset arteriopathy. Vessels are characterized by smooth muscle degeneration as in CADASIL, but without deposition of granular osmiophilic material, the CADASIL hallmark	Nishida et al. (2006) Pedrosa et al. (2015) Reichrath and Reichrath (2020a, b)
	Notch signaling and vascular development	*Notch signaling governs many core processes during embryologic vascular development, from vascular growth and endothelial tip and stalk cell selection to vSMC development*	Scheppke et al. (2012) Segarra et al. (2008) Wang et al. (2012)
		Dll4-mediated Notch signaling	
		Absolutely required for normal arterial specification during embryonic development	
		It is a key regulator of embryonic, postnatal developmental, regenerative, and tumor-sprouting angiogenesis	
		It mediates communication between adjacent endothelial cells (ECs) that lead the sprout formation and adjacent ECs that under Dll4/Notch control remain in the quiescent state in preexisting vasculature or rather proliferate then migrate, forming the trunk of the new vessel	
		Mechanistically, Dll4/Notch enables the selective EC departure from preexisting activated endothelium and organized sprout outgrowth by decreasing the VEGFR2/VEGFR1 ratio and therefore reducing the sensitivity of signal-receiving ECs to VEGF	

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Table 9.1 (continued)

Topic		Clinical and laboratory findings	References (Selection)
		Balanced sprouting is achieved by Dll4-induced "high" Notch signaling and inhibition of sprouting, via suppression of VEGFR signaling in tip cells, which is antagonized in stalk endothelial cells exhibiting JAG1-mediated "low" Notch signaling activity Although Dll4/Notch blockade potentiates the tumor-driven angiogenic response, it inhibits tumor growth due to the formation of immature and poorly functional vessels that result in reduced tumor perfusion	
		JAG1 Loss of vSMCs and severe disruption of angiogenesis found in *JAG1* mutants Systemic knockout of *JAG1* or *NOTCH2* (homozygous) is embryonic lethal in mice at ~E11.5 (because of resulting defects in angiogenesis of the embryonic and yolk sac vasculature) and at ~E10.5 (because of widespread apoptosis), respectively Both endothelial-specific (via Tie1- or Tie2-Cre) and complete deletion of *JAG1* results in embryonic lethality and cardiovascular defects, demonstrating that lack of JAG1 signaling from the vascular endothelium likely results in these anomalies Functions in adults downstream of Dll4/Notch1 signaling to stimulate maturation of vSMCs after injury through P27kip1-mediated inhibition of proliferation JAG1-induced expression of integrin αvβ3, which facilitates binding to a basement membrane-specific von Willebrand factor protein, may mediate the perivascular coverage of newly formed vessels by vSMCs and pericytes Governs angiogenesis-associated sprouting (both gain- and loss-of-function investigations in endothelial cells demonstrate that JAG1 stimulates the sprouting of new tip cells during retinal angiogenesis)	
		NOTCH2: Loss of vSMCs demonstrated in embryos with homozygous hypomorphic Notch2	
Angiogenesis in NMSC	Vascularization in BCCs	Angiogenesis is a characteristic feature of BCCs, that are clinically characterized by the presence of telangiectasias	Wuest et al. (2007)
Notch and Cancer stem cells (CSC)	CSC hypothesis	Postulates that malignant tumors are characterized by a hierarchical structure of different cell subpopulations, including so-called cancer stem–(like) cells or tumor-initiating cells (TIC), that have the capacity for self-renewal and to develop heterogeneous lineages of cancer cells that build up the tumor	Chatterjee and Sil (2019) Espinoza et al. (2013) Quan et al. (2018) Reichrath and Reichrath (2020a, b, c) Shi et al. (2017) Venkatesh et al. (2018)

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Table 9.1 (continued)

Topic		Clinical and laboratory findings	References (Selection)
	CSCs: common features	*Characteristic findings* Slow growth rate Colony- and tumor-forming capacities Altered differentiation Altered migration Altered treatment sensitivity (often resistant to chemotherapy and radiotherapy, thereby resulting in the failure of these conventional therapies Activate Notch and other distinct signal transduction pathways that are of importance for embryonic development and for tissue homeostasis	
		Defining existence and biological function of distinct CSCs Great progress was achieved in different types of human solid tumors through identification of specific subpopulations characterized by expression of distinct surface determinants and other molecular markers, including CD133, CD44, CXCR4, and ALDH1	
		Therapeutic implication Targeting Notch signaling in CSCs represents a promising future direction for the ultimate therapeutic goal to cure cancer	
	CSCs in NMSC	*Maintaining the CSC population in cSCC* Maintaining the CSC population in SCC governed by a complex network of multiple pathways (including WNT, HEDGEHOG, NF-κB, growth factor receptors, RAS-mitogen–activated protein kinase, PI3K–Akt–mTOR, and TP53) important for cellular growth, death or survival, senescence migration, and/or epithelial/mesenchymal differentiation/transition Significant genetic alterations and inactivation or activation of Notch and other pathways relevant for maintaining the CSC population in SCC In primary human cSCC tumors and cell line models, the small and distinct $CD133^+$ subpopulation (live $CD133^+$ cells that form spheroid colonies in vitro and tumors in vivo) differentially expresses stem-like and cancer gene signatures linked to Notch1-mediated NF-κB modulation, NF-κB, and WNT pathways Gene signatures in $CD133^+$ stem cells revealed activation of a highly orchestrated, complex network of multiple pathways, which were linked to Notch and NF-κB signaling and demonstrated sensitivity to genetic and pharmacologic inhibitors of Notch and NF-κB Functional, genetic, and pharmacologic studies uncovered a linkage between Notch1, Wnt, Hedgehog, IKKα, and NF-κB pathway activation in maintaining the $CD133^+$ population and its self-renewal ability in established primary cSCC and cell lines	

(continued)

Table 9.1 (continued)

Topic		Clinical and laboratory findings	References (Selection)
		Crosstalk of Notch signaling with two other major developmental pathways, Hedgehog and Wnt, of importance in many embryological development cascades and in maintaining stemness of stem cells Every single one of these pathways (Notch, Hedgehog, and Wnt) is potent in inducing tumorigenesis, driving tumor progression, and aiding epithelial to mesenchymal transition in malignant cells, apart from maintaining cancer stem cells population inside the tumor tissue Inhibition of all the three pathways individually have resulted in tumor regression, but not optimally, as treatment failure and cancer relapse have been found to occur Wnt pathway is an evolutionarily conserved signaling pathway determining patterning of animal embryos, cell fate, cell polarity, and a substantial role in the origin and maintenance of stem cells IKK and NF-κB signaling: Implicated in promoting tumor cell survival, inflammatory, and angiogenesis responses. How the molecular components of these signaling pathways are orchestrated to comprise the functionally versatile network that governs the induction and regulation of the different phenotypes that are associated with the distinct CSC/TIC subpopulations in cSCC tumors is not well understood Identification of significant molecular/genetic alterations in key pathways that govern the maintenance of the $CD133^+$ CSC phenotype could potentially help identify promising new targets for pharmacological cancer prevention and therapy	
		Therapeutic implications Instead of targeting a single pathway, targeting the crosstalk network could be a better alternative to conventional cancer treatment. Elimination of both tumor cells as well as cancer stem cells implies a reduced risk of relapse. Drugs developed to target these cross-talking networks (e.g., Notch, Wnt, Shh), when used in combinatorial therapy, can hopefully increase the efficacy of the therapy to a very large extent	

Abbreviations (selection): *BCC* Basal cell carcinoma, *cKO* conditional knockout, *CSC* cancer stem cell, *CSL* CBF-1, Su (H), Lag-1-type transcription factor, *DBD* DNA binding domain, *Dll* delta-like, *E* embryonic day, *FGF* fibroblast growth factor, *GOF* gain of function, *Hes* hairy and enhancer of split, *HF* hair follicle, *HPV* human papilloma virus, *Hrt* Hes-related transcription factor, *IP* intermediate progenitor, *IPC* intermediate progenitor cell, *JAG* jagged, *KO* knock-out, *MET* mesenchymal-to-epithelial transition, *MAML* mastermind-like, *MMP* matrix metalloproteinase, *NID* Notch intracellular domain, *NMSC* nonmelanoma skin cancer, *NSC* neural stem cell, *NO* nitric oxide, *OD* oligomerization domain, *PDGF* platelet-derived growth factor, *PTCH* patched, *RBP-J* recombinant recognition sequence binding protein at the J_κ site, *Sema* Semaporin, *SCC* squamous cell carcinoma, *Shh* sonic hedgehog, *TA* transactivation domain, *TLR* toll-like receptor, *VEGF* vascular endothelial growth factor, *vSMC* vascular smooth muscle cell, *Wnt* wingless-related integration site

their corresponding ligands is associated with the stratification of the epidermis (Blanpain et al. 2006). On the molecular level, this process is mediated by enzymatic cleavage of the NID and its translocation to the nucleus, where it associates in keratinocytes of suprabasal cell layers with DNA-binding protein RBP-J to regulate downstream target genes (Kopan and Ilagan 2009; Lowell et al. 2000; Moriyama et al. 2008; Okuyama et al. 2004; Wang et al. 2008).

In skin, the epidermis is maintained throughout life through the proliferation of stem cells and differentiation of their progeny. The innermost (basal) layer of the epidermis consists of proliferative progenitor cells which give rise to multiple differentiating layers, a stratified epithel providing a barrier that keeps the inside of the body moist and protects the body from environmental hazards by physical, chemical, and biological factors, including ultraviolet (UV)-radiation (Massi and Panelos 2012). Investigations using transgenic mice have demonstrated that in contrast to embryonic development of the HF that can be achieved without Notch, its postnatal development requires an intact Notch signaling in two important compartments of the hair, the bulb, and the outer root sheath (reviewed in Aubin-Houzelstein 2012, reviewed in Massi and Panelos 2012). In the hair bulb, Notch governs cell differentiation, ensuring the proper development of every layer of both the hair shaft and the inner root sheath (reviewed in Aubin-Houzelstein 2012, reviewed in Massi and Panelos 2012). Among the many roles played by Notch in the skin and HF, it has to be highlighted that in the bulge, Notch controls a cell fate switch in HF stem cells or their progenitors, preventing them from adopting an epidermal fate (reviewed in Aubin-Houzelstein 2012). Notch function in the skin and HF is both cell autonomous and cell nonautonomous and involves intercellular communication between adjacent cell layers (reviewed in Aubin-Houzelstein 2012, reviewed in Massi and Panelos 2012).

The tightly regulated Notch function depends on a large network of contributing pathways that have also been shown to be of importance for skin carcinogenesis, including Wnt-mediated signals from adjacent epidermal cells and suppressing bone morphogenic protein (BMP)–mediated signals from underlying mesenchymal condensates, which converge to activate Sonic hedgehog (Shh) in the developing hair bud. Loss of Shh signaling widely disturbs this highly regulated epithelial–mesenchymal cross-talk, impairing HF down-growth and maturation in the embryo and distorting homeostasis throughout postnatal skin epithelium (Chiang et al. 1999; Gritli-Linde et al. 2007; Oro and Higgins 2003). Notably, it was shown that epidermal morphogenesis not only precedes but also may be observed independently of Hh signaling (Oro and Higgins 2003).

Notch Signaling and Nonmelanoma Skin Cancer (NMSC)

The physiological/pathophysiological function and the regulation of the Notch pathway in the pathogenesis of human NMSCs (Table 9.1) are at present not completely understood. Previous studies indicate an important role of Notch signaling both for pathogenesis and progression of SCCs and BCCs (reviewed in Reichrath and Reichrath 2012a).

It was demonstrated that in accordance with its function in inducing differentiation of keratinocytes, mice with an experimentally induced epidermal deletion of the Notch1 gene develop extensive epidermal hyperplasia and spontaneously develop BCCs (reviewed in Reichrath and Reichrath 2012a). Consequently, this finding has resulted in the hypothesis that Notch1 may act in the skin as a tumor suppressor (Table 9.1). Moreover, in mice with epidermal inactivation of Notch1, chemical injury promoted the formation of cutaneous lesions representing both BCCs and SCCs, in addition to inducing numerous papillomas. It has been shown that mice expressing a dominant negative MAML1 (DNMAML1) protein to inhibit RBP-J dependent Notch signaling in the epidermis exhibit multiple skin defects including diffuse alopecia, epidermal hyperpla-

sia, and hyperkeratinization (reviewed in Reichrath and Reichrath 2012a). These mice develop spontaneous lesions resembling human SCC and actinic keratoses, but do not develop BCC. In contrast to normal epidermis, keratinocytes and lesional cells from DNMAML1 mutant mice express nuclear ß-catenin and cyclin D1 in a pattern similar to that observed in human cutaneous SCC, suggesting a conserved role for these molecules in SCC (reviewed in Reichrath and Reichrath 2012a). Taken together, these data strongly suggest that functional interactions between Notch signaling, ß-catenin, and cyclin D1 play critical roles in the pathogenesis of cutaneous SCC.

Epidemiology and Clinical Findings of NMSC

BCCs and SCCs, the two major types of NMSC, vary considerably in their clinical presentation, growth patterns, and metastatic capability. In general, most cases of both BCCs and SCCs have a good prognosis, especially when detected at their early stages (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). BCC cells resemble many characteristic features of epidermal basal cells (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). There is evidence that BCCs, at least in part, originate from the basal layer of the outer root sheath of the hair follicle, which closely resembles the interfollicular basal layer of the epidermis with respect to protein expression patterns, including members of the Notch signaling pathway (Table 9.1). BCCs have been described as the least aggressive type of NMSC. They very rarely metastasize and show a low degree of malignancy, despite of the capability of local invasion, tissue destruction, and recurrence (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). It was reported that the prevalence of BCCs and SCCs has increased by 35% and 133%, respectively, over 2 decades. BCCs contribute minimally to the NMSC mortality rate (MR). An incidence rate of 1 case per 14,000,000 for metastatic BCC, and 2 patients per 14,000,000 who die from locally advanced BCC have been reported. In consequence, a MR of 0.02 per 10,000 is to be expected (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). Individual risk factors for BCC include gender, age, immunosuppression, genetic diseases (e.g., Gorlin–Goltz syndrome), and Fitzpatrick skin types I and II (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). Ultraviolet (UV) radiation represents the most important environmental risk factor for BCC pathogenesis, although the precise relationship (chronic or intermittent (sunburn) UV exposure) between UV radiation and BCC development remains controversial (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). BCCs develop predominantly in elderly patients on sun-exposed skin areas. BCCs rarely develop on palmoplantar surfaces and are never found on the mucosa (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). Individuals who develop BCC have an elevated risk of developing new foci of BCC, as well as other types of skin cancer, including melanoma and SCC. Their incidence has increased strongly over time, also reflecting our aging population.

SCCs are characterized by atypical, invasive proliferation of squamous cells, which have the potential to metastasize. SCCs show a considerable potential for recurrence, which depends on many factors, including tumor size, degree of histological differentiation, depth of the lesion, perineural invasion, patient's immune system, and anatomic localization (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). Several risk factors have been reported in SCC patients, including Fitzpatrick skin types I and II, outdoor occupation, human papillomavirus (HPV) types 16, 18, and 31, and several cutaneous genetically inherited skin diseases (including albinism, xeroderma pigmentosum, and epidermodysplasia verruciformis) (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). However, the most important environmental risk factor is UV radiation (artificial and solar) (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). A direct correlation between psoralen and UVA (PUVA) exposure and the incidence of SCC has been reported (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). In most cases, SCCs arise on

sun-exposed areas, with about 55% and 18% of all SCCs presenting on the head and neck area and on the extensor surfaces of the hands and forearms, respectively (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). Nevertheless, up to 13% of SCC cases arise on the legs (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). Actinic keratoses (AKs) represent in situ SCCs, the earliest manifestation of SCCs. Although their prevalence varies according to geographical location and age, AKs are extremely common, showing a prevalence greater than 40% in many adult populations. AKs occur usually on chronically UV-exposed skin. AKs share several pathological features with SCC, and they represent a continuum in a multistep process over the years on chronically sun exposed fair skin. Normal-appearing skin that surrounds AKs may develop AKs, because of the UV exposure and expression of molecular alteration, including p53 mutations. This whole area is today known as "field cancerization." SCCs show a variable metastatic rate of 0.1–9.9% and they account for about 75% of deaths due to NMSC (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014). Although the first-choice therapy is still surgical excision, plenty of alternative approaches have been reported to manage NMSC, including photodynamic therapy (PDT), cryotherapy, and topical imiquimod 5% (Apalla et al. 2017; Didona et al. 2018; Leiter et al. 2014).

The Role of Notch Signaling for Carcinogenesis of Basal Cell Carcinomas

Several studies, including investigations from Thélu and coworkers, compared the expression of Notch receptor family members and their corresponding ligands in BCCs and unaffected and healthy skin (Table 9.1). Expression of Notch1 and its ligands varies in the different layers of the epidermis (Fig. 9.1d–f). In unaffected, healthy skin Notch receptor 1, Dll1 and JAG1 are detectable in the whole epidermis, with pronounced expression of the latter two in the basal layer. Thélu and coworkers demonstrated that the protein expression of Notch receptor 1 and its corresponding ligands Dll1 and JAG1 was markedly reduced in BCC tumor regions as compared with unaffected normal skin (Thelu et al. 2002). Moreover, in that study, all three proteins were undetectable in BCC regions comprised of palisading cells penetrating the dermis. The authors concluded that because in normal human skin Notch receptor 1 and its corresponding ligands Dll1 and JAG1 are detectable in all cell layers of the viable epidermis (with pronounced expression of the latter two in the basal layer), in absence of Notch1, Dll1 and JAG1, missing or reduced Notch signaling activity may cause disordered epidermal terminal differentiation and proliferation (Thelu et al. 2002). It has been speculated that during malignant transformation of BCCs, keratinocytes may enter a pathological status when they neither transcribe Notch receptor family members nor corresponding ligands, thereby resulting in abolishing a fundamental signal for terminal differentiation (Thelu et al. 2002).

In another immunohistochemical study, immunoreactivity for Notch receptor 1 was absent in viable normal epidermis and in BCCs, while Notch receptor 2 and its downstream target gene Hes1 could be detected in both cytoplasm and nuclei of normal skin epithelia (8/8) and, with reduced detection rates, of BCCs (Liu et al. 2008). The authors of this study (Liu et al. 2008) concluded that Notch signaling in normal epidermis may be mediated mainly by Notch receptor 2, and that Notch signaling might have twofold bioactivities: It may benefit normal maintenance/renewal of epidermal cells, while its attenuation may favor epidermal tumor growth due to its tumor inhibitory effects (Bolós et al. 2007; Liu et al. 2008). In contrast to other organs, Notch receptor 1 appears to act as a tumor suppressor in the skin (Table 9.1). It was shown that ablation of Notch receptor 1 from epidermal cells in mice leads to an uncontrolled proliferation of the basal epidermal layer and finally results in BCC-like tumors. The immunohistochemical detection of Notch receptor 1 and JAG1 in BCCs is shown in Fig. 9.1d–f, respectively.

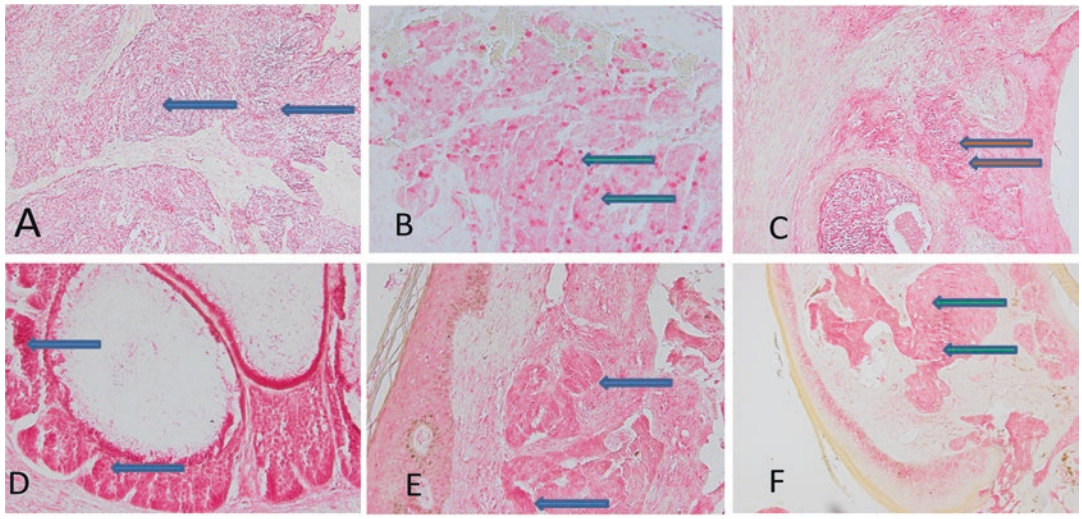

Fig. 9.1 Immunohistochemical detection in cutaneous squamous (**a**–**c**) and basal cell (**d**–**f**) carcinomas of Notch receptors 1 (**b**, **f**;), 2 (**c**;) and corresponding ligand JAG1 (**a**, **d**, **e**;). Note weak-to-moderate cytoplasmic and/or nuclear immunoreactivity (representative tumor areas marked as examples with arrows)

Previous findings indicate that at least a significant subset of BCCs is directly HF derived (Table 9.1) (Shi et al. 2017; Crowson 2006; Grachtchouk et al. 2011; López-Takegami et al. 2016; Peterson et al. 2015). It has been reported that the stem cells of the HF bulge region and adjacent cells represent a potential primary source of BCCs derived from HFs (Shi et al. 2017; Grachtchouk et al. 2011; Peterson et al. 2015). In some regard, HFs and BCCs represent "ordered" and "disordered" variants of skin appendage growths, respectively (Shi et al. 2017). An important property both of BCCs and HFs is the ability of their cells to indefinitely and repeatedly proliferate, a key mechanism that is responsible for maintaining a tumor mass or regular hair fiber production (Shi et al. 2017). It has been speculated that all BCCs, HF-derived or not, may express similar fundamental growth mechanisms to those that regulate HF growth and cycling (Shi et al. 2017). Several specific molecular mechanisms involved in this process of self-renewal, including the Notch and other pathways, including sonic hedgehog (Shh) and Wingless-related integration site (Wnt) signaling, have been found to be active in normal HFs and in BCCs (Thélu et al. 1998; McMahon et al. 2003; Jayaraman et al. 2014; Wuest et al. 2007; Rishikaysh et al. 2014; Shi et al. 2017). Notably, the roles of these signaling networks for BCC growth, particularly the Notch pathway, and their crosstalk, remain until today only poorly understood. Mutations in genes encoding for components of the sonic hedgehog (Shh) and patched (PTCH) signaling pathways are the hallmarks of BCC pathogenesis (Hutchin et al. 2005; Shi et al. 2017). The primary mechanism, by which most BCCs develop, a constitutive activation of the Hedgehog pathway, is a fundamental regulatory mechanism in HF development (Hutchin et al. 2005; Shi et al. 2017). Shh signaling is required for the proliferation and normal cycling of HF epithelium. Modifications of Shh signaling can result in tumor development in tissues of different origins (McMahon et al. 2003). Hyperactivation of the Shh signaling pathway is found in several HF-derived tumors and in BCCs (Dahmane et al. 1997; Oro et al. 1997; Shi et al. 2017). Overexpression of GLI1 and GLI2 gene products have been reported in BCCs, indicating increased Shh signaling (Dahmane et al. 1997; Grachtchouk et al. 2000; Shi et al. 2017). In line with previous reports (Dahmane et al. 1997; Grachtchouk et al. 2000; Shi et al. 2017), enhanced expression of glioma-associated oncogene homolog 1 (GLI1) and GLI2 was demon-

strated in BCCs and in HF root sheaths, compared with normal skin. In these investigations, HF root sheaths exhibited significantly elevated expression levels of GLI1 and GLI2 compared with BCCs. Interestingly, several reports (Jayaraman et al. 2014; Shi et al. 2017) demonstrated the significance of mutations in *NOTCH*1 and *NOTCH*2 in BCCs.

It has been speculated that defining gene expression patterns and pathways in BCCs that are distinct from HF growth and cycling may lead to a better understanding of the abnormal proliferation that these cells undergo in the development of skin cancer. A recent study identified specific molecular mechanisms that are involved in the process of cell self-renewal in HFs and BCCs, including Notch and Hedgehog signaling pathways. Interestingly several key Notch signaling factors showed in that study significant differential expression in BCCs compared with HFs. In that study, a number of genes were uniquely expressed in HFs or BCCs only, indicating that selected Notch pathway genes were differentially activated and inhibited in BCCs, which may be due to positive feedback, and reciprocal negative feedback, from differences in Delta and Notch cell surface expression, or the irregular activation of downstream Notch signaling pathway genes.

Moreover, examination of downstream components of the Notch pathway revealed that the transcription factor RBP-J and downstream target genes of the Hes and Deltex families exhibited a high expression in hair shafts compared with BCCs and normal skin. By contrast, two genes that affect the co-repression of RBP-J, CTBP1, and CREBBP were observed to have a significantly lower expression in HFs compared with BCCs. Deletion of RBP-J from follicular stem cells results in an aberrant cell fate switch that leads to the establishment of epidermal progenitors and basal cells (Yamamoto et al. 2003; Shi et al. 2017). This result, therefore, demonstrated that the Notch/RBP-J signaling pathway is strongly activated in HFs. Since the Notch signaling pathway promotes a stem cell phenotype in skin (Lowell et al. 2000; Shi et al. 2017), the degree of Notch signaling pathway activation may be important for HF stem cell proliferation and differentiation. It has been speculated that the high level of Notch/RBP-J signaling pathway activation may be required for the formation and maintenance of HF.

Importantly, results of this study suggest a reduced expression of downstream genes of the Notch/RBP-J signaling pathway in BCCs. This may allow basal cells to escape from the normal regulation of proliferation that is normally found in the absence of Notch signaling activity, as observed in mammary epithelium cell lineages (Buono et al. 2006; Shi et al. 2017). Loss of RBP-J action in BCCs may promote cells toward a more stem, or progenitor, cell-like status, enabling basal cell tumor growth. Notch signaling via NID translocation into the nucleus, and subsequent binding to the transcription factor RBP-J, may be an important stage of BCC development. As such, RBP-J signaling may be a focus for the introduction of promising, new BCC therapies, as has been suggested for other types of cancer (Garber 2007; Purow 2012; Shi et al. 2017).

Notably, Notch receptor family members 1 and 2 have been reported to exert equivalent or reverse biological effects in cell type-dependent fashions (Weng and Aster 2004). JAG1 plays an important role in the differentiation of keratinocytes, as the activation of Notch pathway triggers terminal keratinocytes differentiation. It was demonstrated that stimulating Notch signaling with JAG1 induced apoptosis of BCC cells by increasing Fas ligand expression and downstream caspase-8 activation. In that study, activation of the Notch signaling pathway by adding exogenous JAG1 into BCC cell culture resulted in increased Fas ligand mRNA and protein expression. This further activated downstream caspase-8 to initiate BCC cell apoptosis. Fas ligand is a type II transmembrane protein that can induce apoptosis upon binding to Fas (Wang et al. 2012; Shi et al. 2017). However, some tumors can decrease Fas expression to resist Fas ligand–mediated T-cell cytotoxicity, and simultaneously upregulate the expression of Fas ligand to induce apoptosis in Fas-expressing T-cells (Satchell et al. 2004; Shi et al. 2017). BCCs strongly express Fas ligand, which may help prevent attack from surrounding immune effector cells,

while also lacking Fas, potentially to make the tumor cells resistant to apoptosis (Erb et al. 2008; Shi et al. 2017). Further investigation is required to characterize the exact role of elevated Fas ligand expression induced by Notch signaling activation by in vivo experiments.

This study adds to the body of evidence that Notch signaling pathway activity is, in contrast to HFs, where it is very strong, suppressed in BCCs. As outlined above, animal models have shown that epidermal skin tumors that spontaneously develop in mice lacking Notch1 display basal cell carcinoma (BCC)–like phenotype. In line with these findings, it has been shown that in BCCs, the protein expression of Notch receptors and corresponding ligands, Dll1 and JAG1 is markedly lowered in tumor regions as compared to healthy epidermis. Interestingly, Thélu and coworkers also reported that they were unable to detect these proteins in the regions with palisading cells penetrating the dermis. In summary, an increasing body of evidence indicates that in the absence of Notch1, Dll1, and JAG1, missing or decreased Notch signaling leads to disorder in epidermal differentiation and proliferation, and promotes formation of BCCs. Impaired Notch signaling is also reported to promote the development of cutaneous squamous cell carcinoma (SCC), as outlined above, and malignant melanoma (MM). In summary, it can be assumed that in contrast to other tissues, Notch seems to function in the skin as a tumor suppressor, as shown by Nicolas et al. (2003).

It has been speculated that pharmacologic modulation of Notch signaling could be a new promising target for the treatment of skin cancer, including BCC, and potentially for hair follicle engineering (Shi et al. 2017). Different therapeutic options are available to treat BCC pharmacologically, including topical immunotherapy (Wuest et al. 2007) and oral treatment with the hedgehog-inhibitor vismodegib. Imiquimod is a small synthetic compound that has been approved for the topical treatment of superficial BCC (sBCC), representing a strong immune response modifier via stimulation of Toll-like-receptor 7 (TLR-7), which is present in plasmacytoid dendritic cells, macrophages, and monocytes (Wuest et al. 2007). This activation of toll-like receptor 7 (TLR7) results in an activation of NF-κB, increased synthesis of proinflammatory cytokines (including interferon-α), and a potent stimulation of antitumor Th1 immunity that finally results in tumor destruction. In a clinical and laboratory investigation, six patients with BCC were evaluated for expression of Notch receptor 1 and corresponding ligands JAG1 and Dll1 before and along with topical treatment with imiquimod using real-time PCR and immunohistochemistry. Interestingly, selective transcriptional upregulation of Notch pathway members (Notch1, JAG1 and Delta1) was detected in that study post-treatment in tumor cells of the BCCs (Wuest et al. 2007). Furthermore, a minor increase of Notch1 protein expression on infiltrating cells as well as strong increase in Jagged1 protein expression was detected in regressing sBCCs post-treatment. Interestingly, Jagged1 is implicated to represent a downstream target of NF-κB activation providing a link between these two signaling pathways. It was speculated that via these mechanisms imiquimod may act as a stimulator of the Notch pathway in sBCC tumor cells by upregulating protein expression of the Notch ligand, JAG1 (Wuest et al. 2007). Moreover, imiquimod may exert tumor suppressor function via induction of Notch signaling, which together with its proinflammatory properties may result in tumor regression.

The Role of Notch Signaling for Carcinogenesis and Progression of Cutaneous Squamous Cell Carcinomas

An Introduction to the Molecular Biology of Cutaneous Squamous Cell Carcinomas: Crosstalk Between Notch and p53 Signaling

Interestingly, cross-regulation among the p53 family members and the Notch signaling pathway has been shown (Missero and Antonini 2014; Roemer 2012). The individual functions of the tumor suppressor p53 (TP53) and its family members, p63 (TP63) and p73 (TP73), and their interactions in the photocarcinogenesis of cuta-

neous SCCs have been extensively investigated (Kouwenhoven et al. 2010; Lang et al. 2004; Lokshin et al. 2007; Missero and Antonini 2014; Yang et al. 2010; Roemer 2012). Many different functions have been assigned to p53, which is often referred to as the "guardian of the genome" because of its ability to prevent mutations (that may be induced upon DNA-damaging stress, including UV-radiation or by other environmental hazards) in the genome by promoting cell cycle exit, senescence or apoptosis (Erb et al. 2008; Hoare and Narita 2018; Lane and Levine 2010; Missero and Antonini 2014; Roemer 2012). The architecture of the p53 protein contains the following domains: transactivation domain (TA), DNA-binding domain (DBD), and oligomerization domain (OD) (Missero and Antonini 2014; Roemer 2012). Due to its crucial role in maintaining genomic stability, inactivation of p53 is the most common event in human cancers, being mutated in over half of human cancers, and often indirectly inactivated via its regulators in other half (Edlund et al. 2012; Lane and Levine 2010; Missero and Antonini 2014; Muller and Vousden 2013; Roemer 2012). Notably, the percentage of C → T transition in the most common mutated amino acids is higher in cSCC as compared to all cancers (consistent with an UV-B radiation signature. No C are found in the codon for R249 (−); data obtained from the UMD TP53 mutation database, release: June 2012_R1; http://p53.fr) (Edlund et al. 2012). Most p53 mutations observed in human tumors fall into the DNA-binding domain (DBD) and inhibit p53 binding to its consensus DNA sequence (Missero and Antonini 2014; Muller and Vousden 2013). As p53 binds DNA as a tetramer, mutant p53 proteins can act with a dominant-negative mechanism on the wild-type protein by heterotetramer formation (Missero and Antonini 2014; Roemer 2012). In addition, some p53 mutants have been shown experimentally to gain novel activities in the absence of a wild-type p53 (Lang et al. 2004; Missero and Antonini 2014; Muller and Vousden 2013; Olive et al. 2004; Roemer 2012).

Each p53 family member encodes several protein isoforms, generated by the presence of alternative promoters, translation initiation sites, and splicing sites. The canonical transactivation domain (TA) present at the N-terminus of the longer protein isoforms is required for transcription of a number of canonical target genes with antiproliferative, pro-senescence or pro-apoptotic, and DNA repair functions. A second internal promoter drives expression of ΔN proteins that lack the TA domain, can exert dominant-negative functions toward the TA proteins and can transactivate a number of specific target genes. This is the case of ΔNp63 and ΔNp73, whereas ΔNp53 (Δ133p53) lacks a small portion of the DNA-binding domain and thus is a selective modulators of some TAp53 functions without binding to canonical p53-binding sites (Missero and Antonini 2014; Roemer 2012). As p53 family members can regulate each other at the transcriptional level (Antonini et al. 2006; Chen et al. 2001; Harmes et al. 2003; Kartasheva et al. 2002; Marcel et al. 2012; Missero and Antonini 2014; Wang and El-Deiry 2006; Roemer 2012), this may in part explain the unbalance expression of their transcripts in cancer.

The structural similarity among the p53 family members and their property to function as tetramers allow heterotetramerization between p63 and p73 isoforms (Davison et al. 1999; Della Gatta et al. 2008; Missero and Antonini 2014; Rocco et al. 2006) and between mutant p53 and p63 or p73 (Di Como et al. 1999; Gaiddon et al. 2001; Missero and Antonini 2014; Strano et al. 2000, 2002; Roemer 2012). The interaction with mutant p53 can lead to p63/p73 inactivation (Di Como et al. 1999; Adorno et al. 2009; Missero and Antonini 2014). Competition for a virtually identical DNA binding site is another crucial level of regulation among p53 family members (Kouwenhoven et al. 2010; Lokshin et al. 2007; Missero and Antonini 2014; Yang et al. 2010). In p53-mutant keratinocytes, p63 and mutant p53 bind to partially overlapping elements, some of which are different from the canonical p63 binding elements found in normal keratinocytes (Martynova et al. 2012; Missero and Antonini 2014). In addition, even when wild-type p53 and p63 bind to the same genomic sites, they regulate largely nonoverlapping gene sets as shown in a lung SCC cell line (Gallant-Behm et al. 2012; Missero and Antonini 2014).

Interestingly, cross-regulation among the p53 family members and the Notch signaling pathway has been shown (Missero and Antonini 2014; Roemer 2012). In keratinocytes, p53 exerts its tumor suppressive function by inducing expression of the pro-differentiation Notch1 and the cell cycle inhibitor p21/CDKN1A, among other target genes (Missero and Antonini 2014; Roemer 2012). A more complex crosstalk exists between p63 and the Notch signaling pathway. p63 directly induces JAG1 and Notch expression, as well as the Notch target IRF6, favoring the initial steps of terminal differentiation (Missero and Antonini 2014). At the same time, p63 suppresses expression of the Notch downstream target genes, p21/CDKN1A and Hes1, sustaining cell cycle progression and repressing late stages of differentiation (Missero and Antonini 2014). Importantly, Notch and IRF6 counteract p63 activity in a negative feedback loop. Relatively little is known about p73, although in other cell types it has been reported to positively regulate JAG1 and JAG2 (Missero and Antonini 2014; Sasaki et al. 2002; Roemer 2012). The p53 family member can potentially regulate each other as indicated and described above.

Notch, p53, and Senescence

Recent findings indicate that Notch signaling is intimately involved in the development of cellular senescence (Hoare and Narita 2018). In contrast to earlier assumptions that thought of cellular senescence as an autonomous tumor suppressor mechanism, cellular senescence has recently been emerging as a phenotype and effector present throughout the life of an organism from embryogenesis to senile decline (Hoare and Narita 2018). Senescent cells exert powerful nonautonomous effects upon multiple players within their microenvironment that they orchestrate mainly through their secretory phenotype (Hoare and Narita 2018). How senescent cells coordinate numerous, sometimes functionally contrasting, outputs through their secretome and/or other mechanisms is still not completely understood, but recent findings indicate a key role of the complex physical and functional interplay between Notch and p53 for the regulation of cellular senescence in both non-malignant and in cancer cells (Hoare and Narita 2018). It has been suggested that a better understanding of the interplay between Notch, p53, and senescence, and how it acts to coordinate the composition and functional effects of the senescence secretome could allow us to develop promising new therapeutics to improve cancer treatment (Hoare and Narita 2018).

The Effects of Notch1 Deletion on Multistage Events in Skin Carcinogenesis

As outlined above, it has been shown convincingly that Notch1 deletion in epidermal keratinocytes causes skin carcinogenesis, while in contrast Notch1 acts in most other tissues as a proto-oncogene (Koch and Radtke 2007). Figure 9.1 shows the immunohistochemical detection of Notch1 in human SCC (b) and BCC (f). The mechanisms underlying the carcinogenesis-promoting characteristics of Notch1-deficient skin have been analyzed in mice with a global or chimeric deletion pattern in their epidermis (Demehri et al. 2009). Results of this study (Demehri et al. 2009) obtained by deleting Notch1 either before or after DMBA treatment in the *K14CreERT* system indicate that loss of Notch1 is not involved in the initiating event of multistage skin carcinogenesis (Demehri et al. 2009; Zoumpourlis et al. 2003). However, it was shown that Notch1 loss acts as a skin cancer-promoting event. In this study, delaying Notch1 deletion in K14CreERT mice until after the tumor-promotion stage of carcinogenesis demonstrated that late deletion of Notch1 contributed to malignant progression of benign papillomas (Demehri et al. 2009), a phenotype that is observed upon loss of p53 but not loss of $p21^{WAF1/Cip1}$, a specific Notch1 target in the skin. In summary, the authors concluded that the main effect of Notch1 loss in skin carcinogenesis is to provide the initiated cells with a proliferative signal to promote tumor growth and proceed to invasive

skin cancer. It has been speculated that this proliferative signal is located downstream of Notch1 loss and could be originated from within the initiated cells, supporting Notch1's role as a classical tumor suppressor in epidermal keratinocytes. As an alternative pathway, it has been hypothesized that this signal could be delivered by the skin microenvironment reacting to Notch1 loss in the epidermis. The experimental system used by Demehri et al. allowed to distinguish between these two possibilities (Demehri et al. 2009). In their study, the chimeric pattern of Notch1 deletion by *Msx2-Cre* created neighboring territories of Notch1-expressing and Notch1-deficient keratinocytes coexisting in the same microenvironment. Examining a large number of tumors isolated from DMBA/TPA-treated Msx2-N1CKO mice clearly demonstrated that tumors comprised mostly (>99%) of Notch1-expressing cells were as likely to form as tumors comprised predominantly of Notch1-deleted cells in the same environment. The authors concluded that Notch1 loss in the epidermis generates a non-cell autonomous signal, promoting tumorigenesis from any initiated cell exposed to the microenvironment conditioned by Notch1-deficient keratinocytes. These results underline the relevance of the microenvironment as an active contributor to tumor development by demonstrating that it can be the primary source of proliferative signals to initiated cells (Demehri et al. 2009).

Tumor Angiogenesis and Cancer Stem Cells: Emerging Therapeutic Targets in NMSC

Tumor Angiogenesis: An Introduction

Malignant tumors, including BCCs and SCCs, consist of a population of constantly and rapidly dividing cancer cells that have lost their ability to control cell division and that progressively accumulate mutations. However, in order to grow and to expand beyond a certain size, malignant tumors need sufficient vascularization (McDougall et al. 2006; Spill et al. 2015). It was reported that malignant solid tumors are unable to grow any more than 2–3 mm in diameter without a sufficient blood supply which corresponds to about 50–100 cells (Nishida et al. 2006). While some scientists believe that the major task of these capillaries is to supply cancer cells with the oxygen and with the essential nutrients that they require, other researchers are convinced that angiogenesis really represents a waste pathway, taking away the biological end products secreted by rapidly dividing cancer cells. To accomplish these needs of supply and/or waste disposal, SCCs and BCCs, like other malignant solid tumors, induce blood vessel growth (angiogenesis), by secreting various growth factors and proteins, that may exert endocrine and paracrine effects (Djokovic et al. 2015; Folkman and Klagsbrun 1987; Folkman 1996). These pro-angiogenic stimulators are then transported to endothelial cells of already existing, nearby located blood vessels, where they cause, via receptor activation, the release of proteolytic enzymes from the vasculature. These enzymes target a particular point on the blood vessel and induce the formation of a characteristic pore that represents the starting point where the new blood vessel will grow from (Nishida et al. 2006). Unlike normal blood vessels, tumor blood vessels are in general dilated with an irregular shape (Gonzalez-Perez and Rueda 2013). It can be emphasized that in either case angiogenesis is an obligate requirement both for transition from small harmless clusters of cells to large life-threatening tumors, and for the metastatic spread of these malignant tumors. Tumor angiogenesis may provide the transport vehicle that enables single cancer cells after these cells have separated from a localized solid tumor have then migrated to and entered these newly build blood vessels, to travel via the bloodstream to distant sites, where they can implant and start the growth of metastases. Evidence from some investigations indicates that the blood vessels in a malignant solid tumor may, in fact, may represent mosaic vessels that are composed not only of endothelial cells but also of tumor cells (Allard et al. 2004). This mosaicity may enable substantial shedding of tumor cells into the vasculature, possibly promoting the distribution of circulating

tumor cells in the peripheral blood of cancer patients (Allard et al. 2004). The subsequent growth of the resulting metastases will also need both the supply of nutrients and oxygen and a waste disposal pathway as obligate requirements (Allard et al. 2004).

The angiogenesis-modulating growth factors produced by tumor cells include fibroblast growth factor (FGF – governs via binding to corresponding cell surface FGF receptors in the presence of heparin proteoglycans a variety of cellular functions such as proliferation and differentiation of all cell types required for building arterial vessels, including endothelial cells and smooth muscle cells), vascular endothelial growth factor (VEGF – stimulates primarily the formation of new capillaries; induces endothelial cells via binding to VEGF receptor-2 a tyrosine kinase signaling cascade that promotes the production of factors that variously stimulate vessel permeability [eNOS, producing NO], proliferation/survival [bFGF], migration [ICAMs/VCAMs/MMPs], and finally differentiation into mature blood vessels), platelet-derived growth factor (PDGF), angiopoietins (Ang – required for the formation of mature blood vessels), matrix metalloproteinases (MMPs – required for the formation of new capillaries; promote the degradation of proteins that keep the walls of blood vessels solid, enabling by this proteolysis endothelial cells to migrate into the interstitial matrix, for example, in sprouting angiogenesis), class 3 semaphorins (SEMA3s – regulate angiogenesis by modulating endothelial cell adhesion, survival, proliferation, migration, and the recruitment of pericytes), and the transmembrane ligand Dll4 of the Notch receptor family. Interestingly, potent negative regulatory effects of Dll4 on angiogenesis have been reported (Hellström et al. 2007; Lee et al. 2016; Lobov et al. 2007; Segarra et al. 2008). One study investigated the effects of DII4 both on tumor vascularity and growth. It was shown that the combined inhibition of VEGF and DII4 in endothelial cells blocks proliferation and sprouting of these cells, thereby blocking angiogenesis throughout the tumor and tumor progression. With this inhibition, cancer growth is stopped very effectively. However, after lifting the blockade, cancer cells will start again to proliferate (Hellström et al. 2007; Lee et al. 2016; Lobov et al. 2007; Segarra et al. 2008). It has been demonstrated that, in contrast to normal blood vessels, tumor blood vessels are dilated with an irregular shape.

As outlined above, pro-angiogenic growth factors, such as FGF and VEGF, can induce capillary growth into malignant tumors. Anti-angiogenic therapies are being employed to fight cancer and malignancies (Folkman and Klagsbrun 1987; Folkman 1996), which require an abundance of oxygen and nutrients to proliferate. Several anti-angiogenic therapeutic approaches can be discriminated regarding their mechanism of action including gene therapy (targeting genes of interest for amplification or inhibition) and protein replacement therapy (which primarily targets pro-angiogenic growth factors like FGF-1 or VEGF). A large number of preclinical studies have investigated efficacy and safety of protein-, gene-, and cell-based therapies in animal models of angiogenesis, including models of cardiac ischemia and of peripheral artery disease. Reproducible, promising, and credible successes in these early in vitro and animal studies led to high enthusiasm that this new therapeutic approach could be rapidly translated to a clinical benefit for millions of patients in the Western world suffering from these disorders. However, several decades of clinical testing both gene- and protein-based treatment modalities designed to stimulate angiogenesis in underperfused tissues and organs, has resulted from one disappointment to another.

Notably, there are still serious, fundamental questions and unsolved problems related to gene therapy that are in part due to the complexity of the genetic and molecular basis of angiogenesis. These obstacles include the potentially increased risk for oncogenesis that may be caused by the viral vectors used for successful and effective integration of the therapeutic genes into the genome of target cells, and for other undesired adverse events (AEs), such as inflammatory autoimmune responses and potential toxicity. In contrast, anti-angiogenic protein therapies are in general based on well-defined, precisely

structured proteins, with previously defined optimal therapeutic doses and with well-known biological effects of the individual protein that may vary for different disease states. On the other hand, protein therapy is also associated with difficulties that include the optimal mode of delivery. Oral, intravenous, intra-arterial, or intramuscular routes of protein administration are not always comparable in their safety and efficacy, because the therapeutically applied protein may be cleared or metabolized before it can enter the target tissue. For many years, there was the assumption that endothelial cells are genetically more stable than cancer cells. This difference in genomic stability may represent an advantage to targeting endothelial cells using anti-angiogenic therapy, as compared to targeting cancer cells, which rapidly mutate and thereby may acquire so called "drug resistance" to therapy with conventional chemotherapy. For this reason, it has been concluded that endothelial cells represent an optimal therapeutic target for gene therapy.

Angiogenesis in BCCs: Lessons Learned from Embryology

Angiogenesis is a characteristic feature of BCCs that are clinically characterized by the presence of telangiectasias (Table 9.1). It has convincingly been shown that in embryology, components of the Notch signaling pathway govern various important aspects of vascular development, from vascular growth and endothelial tip and stalk cell selection to vascular smooth muscle cell (vSMC) development (Table 9.1).

Notably, Dll4/Notch signaling is absolutely required for normal arterial specification during embryonic development and is a key regulator of embryonic, postnatal, developmental, regenerative, and tumor-sprouting angiogenesis (Djokovic et al. 2015; Lobov et al. 2007). It mediates communication between adjacent endothelial cells (ECs) that lead the sprout formation and adjacent ECs that under Dll4/Notch control remain in the quiescent state in preexisting vasculature or rather proliferate and then migrate, thereby forming the trunk of the new vessel (Djokovic et al. 2015). Mechanistically, Dll4/Notch enables the selective EC departure from preexisting activated endothelium and organized sprout outgrowth by decreasing the VEGFR2/VEGFR1 ratio and therefore reducing the sensitivity of signal-receiving ECs to VEGF (Djokovic et al. 2015). Balanced sprouting is achieved by Dll4-induced "high" Notch signaling and inhibition of sprouting, via suppression of VEGFR signaling in tip cells, which is antagonized in stalk endothelial cells exhibiting JAG1-mediated "low" Notch signaling activity (Djokovic et al. 2015). Although Dll4/Notch blockade potentiates the tumor-driven angiogenic response, it inhibits tumor growth due to the formation of immature and poorly functional vessels that result in reduced tumor perfusion (Djokovic et al. 2015).

Because of resulting defects in angiogenesis of the embryonic and yolk sac vasculature, the systemic knockout of Jag1 is embryonic lethal in mice at ~E11.5 (Kiernan et al. 2007, reviewed in Mašek and Andersson 2017; reviewed in Reichrath and Reichrath 2020b). A similar picture is found in homozygous Notch2 knockout mice that are characterized by widespread apoptosis and die at ~E10.5 (Hamada et al. 1999; McCright et al. 2006; reviewed in Mašek and Andersson 2017; reviewed in Reichrath and Reichrath 2020b). The endothelial-specific ablation (via Tie1- or Tie2-Cre) of JAG1 phenocopies systemic Jag1 deletion, demonstrating that a lack of JAG1 signaling from the vascular endothelium likely results in the differentiation defects, loss of vSMCs, and severe disruption of angiogenesis that can be found in JAG1 mutants (Benedito et al. 2009; High et al. 2008; reviewed in Reichrath and Reichrath 2020b). A similar loss of vSMCs has been demonstrated in embryos with homozygous hypomorphic Notch2 (McCright et al. 2001; Wang et al. 2012; reviewed in Reichrath and Reichrath 2020b). Additionally, it has been speculated that the perivascular coverage of newly formed vessels by vSMCs and pericytes is mediated by JAG1-induced expression of integrin αvβ3, which facilitates binding to a basement membrane-specific von Willebrand factor protein (reviewed in Reichrath and Reichrath

2020b; Scheppke et al. 2012). In adults, JAG1 instead functions downstream of Dll4/Notch1 signaling to stimulate maturation of vSMCs after injury through P27kip1-mediated inhibition of proliferation (Boucher et al. 2013; Pedrosa et al. 2015; reviewed in Mašek and Andersson 2017; reviewed in Reichrath and Reichrath 2020b).

JAG1 also governs angiogenesis-associated sprouting; both gain- and loss-of-function investigations in endothelial cells demonstrate that JAG1 stimulates the sprouting of new tip cells during retinal angiogenesis (High et al. 2008; reviewed in Benedito and Hellström 2013; reviewed in Mašek and Andersson 2017; reviewed in Reichrath and Reichrath 2020b). Notably, balanced sprouting is achieved by Dll4-induced "high" Notch signaling and inhibition of sprouting, via suppression of VEGFR signaling in tip cells, which is antagonized in stalk endothelial cells exhibiting JAG1-mediated "low" Notch signaling activity (Benedito et al. 2009; Pedrosa et al. 2015; reviewed in Mašek and Andersson 2017; reviewed in Reichrath and Reichrath 2020b). Although these different aspects of JAG1 and Notch2 signaling have not yet been connected to Alagille or Hajdu–Cheney syndromes (reviewed in Reichrath and Reichrath 2020a, b), they may be of relevance for the severity of these conditions, and the risk for vascular accidents, such as ruptured aneurysms and bleeding (Kamath et al. 2004, 2013; reviewed in Mašek and Andersson 2017; reviewed in Reichrath and Reichrath 2020a, b).

Notch and Cancer Stem Cells: Challenge and Promise to Cure Cancer

An increasing body of evidence indicates an important role of cancer stem cells (CSC) for pathogenesis and progression of many malignancies, including NMSC (Chatterjee and Sil 2019; Espinoza et al. 2013; Quan et al. 2018; Venkatesh et al. 2018). The CSC hypothesis postulates that malignant tumors are characterized by a hierarchical structure that consists of different cell subpopulations, including so called cancer stem–like cells or tumor-initiating cells (TIC), that have the capacity for self-renewal and to develop heterogeneous lineages of cancer cells that comprise the tumor (Quan et al. 2018). Moreover, these cells have in general a slow growth rate, reveal colony- and tumor-forming capacities, as well as altered differentiation, migration, and treatment sensitivity (Quan et al. 2018). They are often resistant to chemotherapy and radiotherapy, thereby resulting in the failure of these conventional therapies (Chatterjee and Sil 2019; Venkatesh et al. 2018). Preventing cancer recurrence and progression by targeting the CSCs is at present a promising perspective to reach the ultimate goal to cure cancer. Although the development of new systemic pharmaceuticals for the treatment of advanced NMSC does not belong to the most urgently needed advances in oncology, the analysis of biology and function of CSC in NMSC has proven to be of particular importance for this rapidly growing field, opening new avenues for the development of new treatment options for a broad variety of different types of cancers (Chatterjee and Sil 2019; Venkatesh et al. 2018). Like any other stem cells, CSCs activate distinct signal transduction pathways, including the Notch signaling pathway, that are of importance for embryonic development and for tissue homeostasis (Chatterjee and Sil 2019; Reichrath and Reichrath 2020a, b, c; Venkatesh et al. 2018). At present, new therapeutic options that target Notch signaling and other pathways that govern stem-cell replication, survival, growth, and differentiation are being developed (Amin et al. 2015; Espinoza et al. 2013; Quan et al. 2018). Notch inhibitors have been introduced to fight cancer and its recurrence either single or in combination with chemotherapy drugs. Targeting Notch and other relevant signaling pathways in CSCs represents a promising future direction for the ultimate therapeutic goal to cure cancer (Espinoza et al. 2013). Great progress in defining the existence and the biological function of distinct CSCs and TICs was achieved in different types of human solid tumors through identification of specific subpopulations characterized by expression of distinct surface determinants and other molecular markers, including CD133,

CD44, CXCR4, and ALDH1 (Quan et al. 2018). Recent studies have revealed in cSCC (and also in HNSCC and lung SCC tumors) significant genetic alterations, involving components of Notch, and inactivation or activation of several other common and distinct canonical pathways important for cellular growth, death or survival, senescence migration, and epithelial/mesenchymal differentiation/transition, including WNT, HEDGEHOG, NF-κB, growth factor receptors, RAS-mitogen–activated protein kinase, PI3K–Akt–mTOR, and TP53 (Chatterjee and Sil 2019; Quan et al. 2018; Venkatesh et al. 2018), but their expression and role in CSC and TIC versus other populations in cSCC have until today not been clearly elucidated. Interestingly, it was demonstrated in primary human cSCC tumors and cell line models that the small and distinct $CD133^+$ subpopulation (live $CD133^+$ cells that form spheroid colonies in vitro and tumors in vivo) differentially expresses stem-like and cancer gene signatures linked to Notch1-mediated NF-κB modulation, NF-κB, and WNT pathways (Quan et al. 2018). Furthermore, characterization of the landscape of gene signatures in these $CD133^+$ stem cells revealed activation of a highly orchestrated, complex network of multiple pathways, which were linked to Notch and NF-κB signaling and demonstrated sensitivity to genetic and pharmacologic inhibitors of Notch and NF-κB. The authors of this investigation concluded that their functional, genetic, and pharmacologic studies uncovered a linkage between Notch1, IKKα, and NF-κB pathway activation in maintaining the $CD133^+$ population and its self-renewal ability in established primary cSCC and cell lines (Quan et al. 2018). IKK and NF-κB signaling has been implicated in promoting tumor cell survival, inflammatory, and angiogenesis responses. However, how the molecular components of these signaling pathways are orchestrated to comprise the functionally versatile network that governs the induction and regulation of the different phenotypes that are associated with the distinct CSC/TIC subpopulations in cSCC tumors are not well understood (Quan et al. 2018). It has been suggested that identification of significant molecular/genetic alterations in key pathways that govern the maintenance of the $CD133^+$ CSC phenotype could potentially help identify promising new targets for pharmacological cancer prevention and therapy (Quan et al. 2018). Wnt pathway is an evolutionarily conserved signaling pathway determining patterning of animal embryos, cell fate, cell polarity, and a substantial role in the origin and maintenance of stem cells. Wnt signaling has been found to crosstalk with Notch, and another major developmental pathway, Hedgehog, in many embryological development cascades and in maintaining stemness of stem cells (Chatterjee and Sil 2019). Research has shown that every single one of these three pathways is potent in inducing tumorigenesis, driving tumor progression, and aiding epithelial to mesenchymal transition in malignant cells, apart from maintaining cancer stem cells population inside the tumor tissue (Espinoza et al. 2013). The role of the crosstalks between Wnt, Hedgehog, and Notch signaling in cancer is under intensive research. Inhibition of all the three pathways individually have resulted in tumor regression, but not optimally, as treatment failure and cancer relapse have been found to occur (Chatterjee and Sil 2019). Hence, instead of targeting a single pathway, targeting the crosstalk network could be a better alternative to conventional cancer treatment. Also, elimination of both tumor cells as well as cancer stem cells implies a reduced risk of relapse. Drugs developed to target these crosstalking networks, when used in combinatorial therapy, can hopefully increase the efficacy of the therapy to a very large extent (Chatterjee and Sil 2019).

Conclusion

Notch signaling controls tissue development during embryonal organogenesis, while in adult tissues it contributes to maintenance of cellular differentiation, proliferation, and apoptosis. Moreover, it governs tumor angiogenesis, maintenance of cancer stem cells, and senescence, thereby playing an important role for pathogenesis and progression of skin cancer. Recent findings demonstrate that Notch signaling has in

cancer a dual action (either as an oncogene or as a tumor suppressor), depending on the tumor type and the synchronous modulation of other intracellular signaling pathways. Further understanding of the pleiotropic roles of the complex network of Notch and other signaling pathways (including hedgehog, Wnt) in BCC and SCC will hopefully finally result in the development of successful new therapies for NMSC.

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Notch Signaling in Thyroid Cancer

10

Rachael Guenter, Zeelu Patel, and Herbert Chen

Abstract

Thyroid cancer is the most common malignancy of the endocrine system with a steadily rising incidence. The term "thyroid cancer" encompasses a spectrum of subtypes, namely papillary thyroid cancer, follicular thyroid cancer, anaplastic thyroid cancer, and medullary thyroid cancer. Each subtype differs histopathologically and in degrees of cellular differentiation, which may be in part due to signaling of the Notch pathway. The Notch pathway is an evolutionarily conserved signal transduction mechanism that regulates cell proliferation, differentiation, survival, stem cell maintenance, embryonic and adult development, epithelial–mesenchymal transition, and angiogenesis. Its role in cancer biology is controversial, as it has been shown to play both an oncogenic and tumor-suppressive role in many different types of cancers. This discordance holds true for each subtype of thyroid cancer, indicating that Notch signaling is likely cell type and context dependent. Whether oncogenic or not, Notch signaling has proven to be significantly involved in the tumorigenesis of thyroid cancer and has thus earned interest as a therapeutic target. Advancement in the understanding of Notch signaling in thyroid cancer holds great promise for the development of novel treatment strategies to benefit patients.

Keywords

Thyroid cancer · Notch signaling · Notch pathway · Papillary thyroid cancer · Follicular thyroid cancer · Medullary thyroid cancer · Anaplastic thyroid cancer · HDAC inhibitors · Notch signaling modulation

Introduction

Thyroid cancer encompasses a wide range of malignant subtypes that can differentially arise from various cell types within the thyroid gland. These subtypes include papillary, follicular, anaplastic, and medullary thyroid cancer. The incidence of these cancers is steadily increasing, with a parallel increase in mortality rates as well (Morris et al. 2013; Furuya-Kanamori et al. 2016; Mao and Xing 2016). Localized disease is often curable with surgery, but patients with metastatic disease have limited treatment options.

Since its discovery in the early twentieth century, Notch signaling has proven to be a critical pathway in mammalian development by regulating

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cell fate decisions, proliferation, differentiation, and survival (Dexter 1914; Mohr 1919; Guruharsha et al. 2012). Four transmembrane receptor isoforms, termed Notch1–4, are capable of binding to five different ligands (Delta-like-1, −2, −4, Jagged1, and Jagged2) in a juxtracrine manner (Takebe et al. 2014). This interaction initiates intracellular cleavage events that ultimately lead to the transcription of specific target genes (reviewed in other chapters).

The role of Notch signaling in cancer is particularly complex, as it acts as either an oncogene or a tumor suppressor. An oncogenic role of Notch signaling has been reported in breast cancer (Reedijk et al. 2005), colon cancer (Sikandar et al. 2010), T-cell acute lymphoblastic leukemia (T-ALL) (Ellisen et al. 1991), chronic lymphocytic leukemia (CLL) (Fabbri et al. 2011; Puente et al. 2011), non-small cell lung cancer (Westhoff et al. 2009), pancreatic adenocarcinoma (Hanlon et al. 2010), clear cell renal cell carcinoma (CCRCC) (Sjölund et al. 2008), and in gliomas (Dantas-Barbosa et al. 2015). On the other hand, Notch signaling has been shown to limit tumorigenicity. This effect was first described in keratinocytes (Nickoloff et al. 2002), and has since been observed in other cancers, including prostate cancer (Shou et al. 2001), small cell lung cancer (Sriuranpong et al. 2001), pancreatic neuroendocrine cancer (Nakakura et al. 2005; Kunnimalaiyaan et al. 2005), hepatocellular carcinoma (Qi et al. 2003; Viatour et al. 2011), cervical cancer (Talora et al. 2002), B-cell malignancies (Zweidler-McKay et al. 2005), myeloid Leukemia (Klinakis et al. 2011), head and neck squamous cell carcinoma (Stransky et al. 2011), and neuroblastoma (Zage et al. 2012).

Targeting the Notch pathway for cancer treatment continues to attract interest. In thyroid cancer, the importance of Notch signaling is only beginning to be understood. In this chapter, the diverse functions of Notch signaling in thyroid cancer will be discussed, along with current strategies used to target and modulate Notch signaling as a possible anticancer therapy.

A Spectrum of Thyroid Cancer Subtypes (Fig. 10.1)

Thyroid cancer is the most common malignancy of the endocrine system with an increasing global incidence (Zhang et al. 2017; Seib and Sosa 2019; Schneider and Chen 2013; Powers et al. 2019). The heterogenous clinical presentations and genetic profiles of thyroid cancer can make this disease complex in nature. The term "thyroid cancer" encompasses a range of subtypes that originate from different cell types within the thyroid, namely, follicular thyrocytes and the parafollicular C-cells. Across the various thyroid cancer subtypes, the degree of cellular differentiation has a strong influence on disease progression, treatment strategies, and overall patient survival (Jung et al. 2017; Yu et al. 2016; Yuan et al. 2019). As these cancers dedifferentiate, they tend to become more aggressive and gain lethality (Ragazzi et al. 2014; Cooper DS 2006; Gallo et al. 2018).

Well-differentiated tumors of the thyroid fall on one end of the spectrum and include thyrocyte-derived papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC). These subtypes account for over 90% of thyroid cancer cases and are generally associated with good prognoses and high survival rates (Yu et al. 2016; Zhang et al. 2017; Yamashita et al. 2013; Xiao et al. 2009; Jung et al. 2017; Choi et al. 2016). FTC tends to be more aggressive than PTC; although local and distant metastases have been reported in both subtypes, leading to poor clinical outcomes (Xiao et al. 2009; Lin et al. 2004).

The other end of the subtype spectrum includes less-differentiated thyroid cancers, meaning that the cells lack thyroid cell–specific characteristics. One of the most lethal human cancers, anaplastic thyroid cancer (ATC) falls within this group. ATC causes over 50% of thyroid cancer deaths and carries a 1-year survival rate of less than 20% (Chen et al. 2008; Hsu et al. 2014; Smallridge and Copland 2010). Another subtype that lacks differentiation is medullary thyroid cancer (MTC). This subtype originates

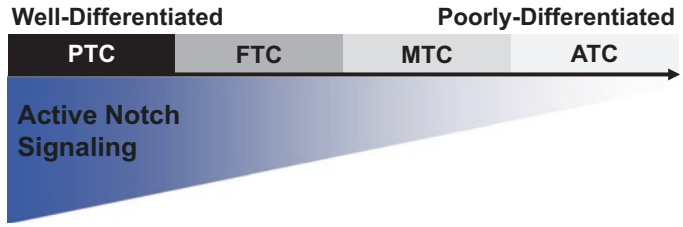

Fig. 10.1 Hypothesized relationship between Notch signaling and thyroid cancer differentiation. Although the role of Notch signaling is controversial, a majority of investigative efforts have shown that as thyroid cancer cells lose differentiation, Notch signaling also decreases. The directionality of this relationship is not clear, but the canonical role of Notch signaling in mammals suggests that a loss of Notch signaling would contribute to a reduction in cellular differentiation

from the parafollicular C-cells of the thyroid and is classified as a neuroendocrine neoplasm (Jaskula-Sztul et al. 2011; Lou et al. 2018; Roy et al. 2013). Localized cases of MTC are often curable, but patients with distant metastases have a 5-year survival rate less than 50% (Lou et al. 2018).

There is an evident and urgent need to improve patient outcomes by developing effective therapeutic options for each thyroid cancer subtype. Among each of them, the Notch signaling pathway consistently emerges as a therapeutic target due to its frequently observed dysregulation (Hsu et al. 2014; Takebe et al. 2014). In this chapter, the highly controversial role and current breadth of research available regarding Notch signaling in each subtype of thyroid cancer will be summarized.

Notch Signaling in Papillary Thyroid Cancer

Papillary thyroid cancer (PTC) is the most common subtype of thyroid cancer and is considered to be well differentiated. The role of Notch signaling in PTC is not clearly defined, as it has been discordantly reported as both oncogenic and tumor suppressive.

In 2011, Park et al. analyzed tissues from patients with PTC and found that the IHC expression of the Notch1 receptor correlated with the increased presence of nodal metastases, extrathyroidal extension, and greater tumor size. However, they found no correlation between the presence of the Notch3 receptor and clinical-pathological factors. Therefore, the authors concluded that in PTC, Notch1 expression is correlated with poor prognostic factors, but Notch3 expression is not. That same year, Geers et al. (2011) found that Notch1 is expressed in normal thyrocytes and has even higher expression in PTC. In support of the notion that PTC overexpresses Notch receptors, additional studies showed that the upregulation of Notch1 expression was observed in both human PTC tissue and a transgenic mouse model of PTC (Yamashita et al. 2013; Gallo et al. 2018). Likewise, greater Notch1 and Notch2 expression was also observed in a PTC cell line when compared to normal thyrocytes (Gallo et al. 2018). Interestingly, the Notch3 and Notch4 isoforms were reported to have low, variable expression levels in PTC cell lines (Gallo et al. 2018). Taken together, these studies conclude that PTC overexpresses the Notch1 and Notch2 isoforms, but the expression of the Notch3 and Notch4 isoforms in PTC is variable. The higher levels of Notch1 and Notch2 in PTC appear to be correlated with more aggressive disease factors.

A recent meta-analysis of 421 patients with PTC conducted by Yuan et al. revealed a significant correlation between an upregulation of Notch1 signaling and the presence of nodal metastasis, tumor size, clinical stage, and capsu-

lar invasion (Yuan et al. 2019). Importantly, the authors concluded that greater Notch1 signaling in PTC could contribute to a poor prognosis for patients, emphasizing an oncogenic role of Notch signaling in PTC. A separate study performed an extensive immunohistochemical analysis of Notch1 expression in 106 thyroid neoplasms and similarly discovered that Notch1 expression was associated with PTC; however, somewhat contrary to the previously mentioned studies, this study found that Notch1 expression was seen exclusively in the tumor cells, highlighted by results that showed normal thyroid tissues were consistently negative for Notch1 expression (Piana et al. 2019).

In contrast to a possible oncogenic role of Notch signaling in PTC that was been discussed in this chapter thus far, several studies have shown that a reactivation, or an overexpression, of the Notch pathway mediates a tumor-suppressive effect by suppressing the growth of PTC cells. Such studies showed that Notch1 is highly expressed in normal human thyroid tissue, but minimally present in both resected human metastatic PTC tissue and metastatic PTC cells in vitro (Xiao et al. 2009; Yu et al. 2016). Furthermore, a significantly higher rate of PTC recurrence was observed in patients with low levels of Notch1 expression (Yu et al. 2016). In a study conducted by Xiao X and colleagues, the pharmacological induction of Notch1 protein expression resulted in a dose-dependent growth reduction of PTC cells in vitro. In accordance with these observations, the reactivation of Notch signaling in PTC has been speculated as a therapeutic strategy.

The role of Notch signaling in PTC lacks a definitive consensus, thereby demanding further investigation. This disparity suggests that the mechanisms of Notch signaling in PTC are contextually dependent, with the degree of cellular differentiation playing a potentially critical role in the determination of how Notch signaling affects cells. Additionally, it appears that the individual Notch receptor isoforms can have different implications on PTC cells, although the direct mechanisms are yet to be resolved.

Notch Signaling in Follicular Thyroid Cancer

Follicular thyroid cancer (FTC) carries a similar degree of well differentiation as PTC, although these two subtypes are histopathologically different. The primary behavior of Notch signaling in FTC has been documented as tumor suppressive. Current research has shown that in comparison to normal thyroid tissue, FTC cells have a lower level of Notch receptor expression, along with lower levels of the Notch signaling target gene, *Hes1*, thus suggesting minimal pathway activity (Ferretti et al. 2008; Xiao et al. 2009; Somnay et al. 2017). In fact, one study discovered that the expression level of the Notch3 receptor was highest in both normal human thyroid tissue and in normal thyroid cells grown in vitro, when compared against resected human FTC tissue and FTC cells grown in vitro (Somnay et al. 2017). Moreover, it has also been shown that metastatic FTC expresses even lower levels of Notch1 than primary FTC, even when comparing metastatic versus primary cells taken from the same patient (Xiao et al. 2009). In summary, these studies demonstrate that normal thyroid cells retain expression of the Notch1 and Notch3 receptors, but FTC lacks expression of Notch1 and Notch3 (Notch2 and Notch4 have yet to be investigated in FTC). These findings have led to the hypothesis that upregulating Notch signaling in FTC could cause tumor suppression.

In order to modulate Notch pathway activity in FTC for an anticancer effect, efforts have been made to increase, or reinstate, Notch signaling in these cells. Overexpressing the intracellular domain of either Notch1 or Notch3 by in vitro plasmid transfection demonstrated the ability to reduce FTC growth while simultaneously increasing markers of thyrocyte differentiation (Ferretti et al. 2008; Somnay et al. 2017). In a complimentary fashion, knocking down the expression of the Notch3 intracellular domain in FTC cells using silencing ribonucleic acid (siRNA) led to an expected increase in cell migration and reduction of thyrocyte differentiation markers (Somnay et al. 2017). This tumor-

suppressive effect was also observed after pharmacological modulation of Notch signaling. Several compounds classified as histone deacetylase (HDAC) inhibitors, which result transcriptional upregulation due to chromatin relaxation, have been employed to increase the expression of the Notch1 receptor (Xiao et al. 2009). A higher level of Notch1 expression in FTC was subsequently linked to various anticancer effects. This included a reduction in FTC cell proliferation in vitro, growth inhibition by cell cycle arrest, and increased apoptosis exemplified by the induction of apoptotic markers (Xiao et al. 2009; Yu et al. 2016).

Current evidence heavily supports a tumor-suppressive role of Notch signaling in FTC. Studies that investigated Notch signaling in tumor samples from patients with FTC found that lower expression levels of Notch receptors were associated with more aggressive, less-differentiated disease and that Notch expression could potentially be used as a prognostic marker to predict patient outcomes (Yu et al. 2016; Somnay et al. 2017).

Notch Signaling in Medullary Thyroid Cancer

The third subtype of thyroid cancer is medullary thyroid cancer (MTC), a malignancy of the neuroendocrine system. This subtype can be characterized by hormone secretions that cause debilitating side effects in patients (Greenblatt and Chen 2007; Cook et al. 2010). Unlike the controversial role of Notch signaling described in PTC, the role of Notch signaling in MTC has been consistently reported to be tumor suppressive. Many studies have shown that the expression of Notch1 is downregulated in both tumor tissue from patients with MTC and in MTC cell lines (Cook et al. 2010; Jaskula-Sztul et al. 2011; Ning et al. 2008). More specifically, MTC features an upregulation of achaete-scute homolog-1 (ASCL1), a transcription factor critical for normal development of parafollicular cells and is transcriptionally repressed by *Hes1*, a target gene of the Notch pathway (Jaskula-Sztul et al. 2011; Ning et al. 2008).

To investigate the effects of Notch1, and subsequently ASCL1 expression, a doxycycline-inducible-Notch1-intracellular domain model was developed in vitro to study MTC (Jaskula-Sztul et al. 2011). The artificial induction of Notch1 resulted in a dose-dependent downregulation of ASCL1 expression, a decrease in MTC cell proliferation, and a reduction in the secretion level of a hormone named calcitonin (Jaskula-Sztul et al. 2011). This effect was also observed in vivo, where MTC xenografts that underwent Notch1 induction by doxycycline had an average reduction in tumor volume by 57% when compared to MTC xenografts without Notch1 induction (Jaskula-Sztul et al. 2011).

Interestingly, the extent of MTC growth inhibition was directly proportional to the amount of Notch1 protein present. The mechanism of MTC cell growth inhibition upon Notch1 activation has been speculated to occur through cell cycle arrest at the G1/S phase due to the upregulation of p21, phospho-Cdc2, and cyclin D1 (Jaskula-Sztul et al. 2011). Accordingly, the mechanism by which ASCL1 protein decreases upon Notch1 activation may be due to transcriptional silencing of ASCL1, likely by *Hes1*, which is known to transcriptionally and translationally inhibit ASCL1 (Chen et al. 1997; Sriuranpong et al. 2002; Sueda et al. 2019; Jaskula-Sztul et al. 2011). Notably, the overexpression of the Notch1 intracellular domain was also correlated with a dose-dependent decrease in chromogranin-A (CgA), a hormone known to be widely expressed in neuroendocrine neoplasms (Jaskula-Sztul et al. 2011). Therefore, one could conclude that activating Notch signaling through the Notch1 receptor in MTC, where Notch signaling is innately low, has the potential to decrease the oncogenic neuroendocrine phenotype.

In addition to the Notch1 receptor, the Notch3 receptor has also been identified a potential therapeutic target in MTC. In 2017, Lou I and colleagues created a doxycycline-inducible MTC cell line to specially overexpress the Notch3 intracellular domain. When this

model was tested in vivo, induction of Notch3 did not prevent tumor formation, but did show an anti-proliferative effect in which led authors to conclude that activating Notch signaling through the Notch3 receptor could serve as a potential treatment option for patients with metastatic MTC (Lou et al. 2018). In summary, currently available research has demonstrated that Notch signaling is diminished in MTC and upregulating the pathway can reduce the oncogenic attributes of MTC.

Notch Signaling in Anaplastic Thyroid Cancer

Anaplastic thyroid cancer (ATC) is the least differentiated subtype and harbors the least favorable patient outcomes out of all thyroid cancer subtypes. Most of the currently available research reports the Notch pathway as tumor suppressive in ATC. This section will describe what is known about Notch signaling in ATC.

The first step in elucidating the role of Notch signaling in ATC warrants an exploration into the basal expression levels of the various Notch receptor isoforms. To this extent, multiple studies report a loss of Notch1 expression in ATCs (Ferretti et al. 2008; Patel et al. 2014; Yu et al. 2013b). Moreover, it has been shown that as thyroid cancer cells become less differentiated, they also lose Notch1 expression (Ferretti et al. 2008; Somnay et al. 2017; Piana et al. 2019). Such evidence has led to the conclusion that a lack of Notch signaling is correlated with more aggressive thyroid tumors, such as ATC. In further support of this conclusion, Notch1 has been experimentally overexpressed in ATC cells with results showing a reduction in cell proliferation and migration, along with an increase, or reinstitution, of thyrocyte differentiation markers (Ferretti et al. 2008; Yu et al. 2013b; Patel et al. 2014). These studies that have described a tumor-suppressive mechanism of Notch signaling in ATC suggest that the Notch pathway may not be solely responsible for causing ATC progression, but it definitely acts to suppress growth and is involved with cellular differentiation. Therapeutically activating Notch signaling has been thought to be a potentially effective treatment strategy for ATC, which is currently considered to be an incurable disease.

The investigation into Notch signaling in ATC is far from complete. Despite the evidence previously described, one paper has reported an oncogenic role of the Notch pathway in ATC. This paper contradicts the aforementioned ATC studies in two ways. The first is by stating that ATC expresses higher levels of both the Notch1 receptor and the active Notch1 intracellular domain when compared to PTC (Kim et al. 2017). The second is that the knockdown of Notch1, and consequently Notch signaling, reduced ATC cell proliferation and migration (Kim et al. 2017). When Notch1 expression was knocked down, the reduction of ATC cell growth and migration could be confounded by the reported greater amount of cell death. The majority of available literature dedicated to understanding the role of Notch signaling in ATC shows agreement with each other. There is, however, a single publication that directly opposes the studies that have shown that the overexpression of Notch1 was significantly associated with decreased cell growth and migration in ATC cells (Ferretti et al. 2008; Yu et al. 2013b; Patel et al. 2014). This discordance necessitates further investigation of Notch signaling in ATC.

Modulating Notch Signaling in Thyroid Cancer Using Natural Compounds

Although the role of Notch signaling in thyroid cancer has yet to be clearly defined, it clearly holds significance. As previously discussed, many studies have shown that Notch signaling is lower in thyroid cancer cells and the reactivation of the pathway yields a tumor-suppressive effect. Based on this observation, various natural compounds have been identified that induce Notch signaling in thyroid cancer. In this section, several different compounds shown to modulate Notch signaling as an anticancer mechanism will be discussed in detail.

The first compound that will be discussed herein is thiocoraline, a thiodepsipeptide intercalator found in marine bacteria. This compound has demonstrated cytotoxic effects in different cancers, including lung, breast, colon, renal, and melanoma (Romero et al. 1997; Erba et al. 1999; Negri et al. 2007; Wyche et al. 2014). The precise mechanism of action in which thiocoraline works is not fully elucidated, but several studies have shown that it can induce G1 cell cycle arrest. However, it has also been shown to activate the Notch pathway in MTC cells as demonstrated by elevated levels of Notch1 and Notch2 after treatment (Rashid et al. 2018; Tesfazghi et al. 2013). This activation was further supported by increased mRNA levels of the downstream targets: *Hey1, Hey2, Hes1*, and *Hes2* (Rashid et al. 2018; Tesfazghi et al. 2013). Conclusively, thiocoraline treatment lead to antiproliferative effects on MTC cells in vitro, as well as the downregulation of markers correlated with poor prognoses, which could be attributed to the activation of the Notch signaling pathway (Tesfazghi et al. 2013).

Another compound that has demonstrated a Notch-activating effect is resveratrol, a polyphenolic compound found naturally in grapes and berries, along with other plants (Yu et al. 2013b). Resveratrol has primarily been studied for Notch pathway induction in ATC cells. In vitro studies revealed that it was capable of suppressing growth in ATC cells through cell cycle arrest in addition to causing apoptosis (Yu et al. 2013b). Moreover, resveratrol activated Notch1 signaling but no there was no apparent change in the active forms of Notch2 or Notch3, suggesting that resveratrol is likely specific to regulating the Notch1 isoform. Resveratrol also increased the transcription of the thyrocyte differentiation markers: *TTF1, TTF2*, *PAX8*, and the sodium/iodide symporter (*NIS*). Finally, resveratrol was tested in vivo and significantly reduced the tumor volume of ATC xenografts as compared to untreated control groups (Yu et al. 2013b).

In the context of ATC, these cells were also sensitive to induction of Notch signaling upon treatment with a compound named hesperetin (Patel et al. 2014). Hesperetin is a naturally occurring flavanone found in citrus fruits. Similar to resveratrol, hesperetin has been shown to activate Notch1 signaling, cause cellular apoptosis, and induce thyrocyte differentiation. More specifically, in vitro studies on an ATC cell line resulted in growth inhibition after treatment with hesperetin. Analysis of apoptotic markers suggested that the primary mechanism of the observed reduction in growth was attributed to apoptosis. Furthermore, hesperetin was shown to dose-dependently increase the amount of Notch1 protein and the downstream markers *Hes1* and *Hey1* present in ATC cells, indicating a functional increase in Notch signaling. This compound, similar to resveratrol, dose-dependently increased thyrocyte-specific transcription factors, namely *TTF1*, *TTF2*, *PAX8*, *TSHR*, and *NIS* (Patel et al. 2014).

Another compound shown to activate Notch signaling in ATC is chrysin (Yu et al. 2013a). Chrysin is a natural flavonoid found in honey that inhibited ATC cell growth in a dose- and time-dependent manner. In vitro experiments showed that chrysin activated the Notch1 signaling pathway at micromolar concentrations. In vivo, chrysin reduced ATC xenograft volume. The tumors showed markers of apoptosis, indicating chrysin likely caused cell death through this mechanism (Yu et al. 2013a).

ATC is particularly difficult treat for many reasons, including the fact these tumors do not concentrate radioiodine due as a result of their undifferentiated features (Patel et al. 2014). During the process of dedifferentiation, thyrocytes lose the expression of the TSH receptor and thyroglobulin, making the cells unable to absorb radioiodine (Xiao et al. 2009). The loss of radioiodine is strongly associated with larger tumors and the presence of distant metastases in thyroid cancer (Xiao et al. 2009; Schlumberger 1998). Therefore, a potential treatment strategy would include inducing redifferentiation in these cells to promote the uptake of iodide. In fact, this strategy has previously been employed by using retinoic acid to induce redifferentiation to promote radioactive iodide uptake in thyroid cancer, as well as breast cancer (Kogai et al. 2006; Schmutzler et al. 1997; Grünwald et al. 1998; Grüning et al. 2003).

The final class of compounds shown to modulate Notch signaling in thyroid cancer are histone deacetylase inhibitors (HDAC inhibitors), specifically valproic acid (VPA), suberoylbishydroxamic acid (SBHA), and trichostatin A (TsA) (Damaskos et al. 2016; Adler et al. 2010; Spartalis et al. 2019; Jang et al. 2015). In preclinical studies, these HDAC inhibitors decreased thyroid cancer cell growth and induced Notch signaling, primarily shown through an increase in the Notch1 isoform. Due to the abundance of evidence showing the efficacy of HDAC inhibitors to have an anticancer effect in thyroid cancer, these compounds have been further tested in several clinical trials. The results of such trials will be discussed in the next section.

Clinical Impact of Notch Signaling in Thyroid Cancer

Most well-differentiated thyroid cancers have good prognoses and low mortality rates. These well-differentiated and often localized tumors are usually curable by surgery, radioiodine ablation, chemotherapy, or thyroid-stimulating hormone (TSH)–suppressive therapy (Hsu et al. 2014). However, these treatments are ineffective for patients with poorly differentiated or metastatic thyroid cancer, making most treatment options palliative (Kim et al. 2017; Smallridge and Copland 2010; Hsu et al. 2014).

Beyond preclinical studies, targeting and modulating the Notch pathway in thyroid cancer has also been explored in a clinical setting. Notch-targeting mechanisms that have been explored clinically include: anti-Notch receptor antibodies, silencing RNA against Notch genes, and γ-secretase inhibitors (GSIs) to prevent the S3 cleavage of the Notch intracellular domain (Jin et al. 2016). One study showed that directly inhibiting the Notch1 receptor using a monoclonal antibody against the negative regulatory region inhibited cancer cell growth, but simultaneously inhibiting the Notch1 and Notch2 receptors caused severe gastrointestinal toxicities in vivo (Wu et al. 2010). It is important to note that this study was not conducted in any thyroid cancer models. A reversible, noncompetitive, and selective small molecule designed as a GSI was administered to a patient with advanced PTC. This patient achieved complete remission with decreased levels of HES4 in peripheral blood, indicating a downregulation of Notch signaling (Messersmith et al. 2015).

Several Phase I and Phase II clinical trials have been conducted to explore the efficacy of HDAC inhibitors in patients with advanced thyroid cancer. All of these trials used HDAC inhibitors to reduce tumor burden and induce differentiation in the thyroid cancer cells, in addition to the goal of increasing radioactive iodide uptake.

One Phase II clinical trial investigated the use of the HDAC inhibitor valproic acid (VPA) in patients with advanced-stage thyroid cancer of follicular origin that had not responded to conventional treatments (Nilubol et al. 2017). VPA is currently approved by the Food and Drug Administration (FDA) for the treatment of epilepsy and bipolar disorder. Numerous preclinical studies have shown that VPA is capable of reducing thyroid cancer cell growth and inducing redifferentiation for increased radioiodine uptake, possibly due to the upregulation of Notch signaling (Fortunati et al. 2004; Greenblatt et al. 2008; Shen et al. 2005; Catalano et al. 2005, 2006). Two objectives cited for this study were to first determine if VPA could reduce tumor growth and cause cancer cell death, and secondly decide if VPA increased the uptake of radioiodine by thyroid cancer cells. The results of the trial were disappointing, in that none of the 10 patients who completed 10 weeks of treatment had increased radioiodine uptake by their thyroid tumors. No partial or complete responses were observed, and 6 of the 10 patients had disease progression. The conclusion of this study was that VPA does not have anticancer activity in patients with advanced thyroid cancer originating from follicular thyroid cells. Likewise, VPA does not increase radioiodine uptake in these patients.

Other clinical trials have assessed the effect of HDAC inhibitors on thyroid cancer with variable results. In a Phase I clinical trial using vorinostat (suberoylanilide hydroxamic acid [SAHA]), one patient had a partial response and five patients, including one patient with MTC, had stable dis-

ease (Kelly et al. 2005). Notably, this study also reported that one patient had higher radioiodine uptake after receiving the drug. A subsequent Phase II clinical trial also investigating the efficacy of vorinostat in patients with advanced thyroid cancer showed that none of the 19 patients enrolled had neither a partial nor complete response (Woyach et al. 2009). This same study examined 16 patients with differentiated thyroid cancer and 3 patients with MTC with the overall conclusion that vorinostat is not effective for the treatment of advanced thyroid cancer. Similarly, a Phase II clinical trial that tested another HDAC inhibitor, Romidepsin, also ended with disappointing results when none of the 16 patients with advanced thyroid cancer had neither a partial nor complete response, in addition to only 2 of the 16 patients having increased radioiodine uptake (Sherman et al. 2013). Conclusively, HDAC inhibitors have shown promise in preclinical settings, but have not manifested any beneficial clinical effects. Taking these results into consideration, in addition to the strong evidence that HDAC inhibitors activate the Notch pathway, a deeper understanding of Notch signaling in thyroid cancer could lead to improved studies and clinical trials that yield better outcomes for patients.

Conclusion

Notch signaling is a multifunctional pathway that canonically plays a critical role in mammalian development. It has also emerged as a key player in many different cancers with various roles ranging from carcinogenesis to cancer cell differentiation. To layer the complexity of Notch signaling, it is widely accepted that the role of this pathway is cell-type and context dependent while also harboring variations in outcomes between the four different receptors and five different ligands. To this extent, Notch signaling has been described to be oncogenic (Reedijk et al. 2005; Sikandar et al. 2010; Ellisen et al. 1991; Fabbri et al. 2011; Puente et al. 2011; Westoff et al. 2009; Hanlon 2010; Sjölund et al. 2008; Dantas-Barbosa et al. 2015) and antioncogenic in various types of cancer (Nickoloff et al. 2002; Shou et al. 2001; Sriuranpong et al. 2001; Nakakura et al. 2005; Kunnimalaiyaan et al. 2005; Qi et al. 2003; Viatour et al. 2011; Talora et al. 2002; Kunnimalaiyaan et al. 2006; Morimura et al. 2000; Zweidler-McKay et al. 2005; Klinakis et al. 2011; Stransky et al. 2011; Zage et al. 2012; Rangarajan et al. 2001). The discrepancy between tumor-promoting and tumor-reducing effects of Notch signaling exists among the different subtypes of thyroid cancer.

The most differentiated thyroid cancer subtype, PTC, is most commonly reported to have a high level of Notch signaling (Yamashita et al. 2013; Gallo et al. 2018; Yuan et al. 2019; Piana et al. 2019). The elevated activity of Notch in this subtype could be in part due to the well differentiated status of the cells, supported by the widely accepted concept that Notch signaling can directly promote cellular differentiation (Lobry et al. 2011). Although, a handful of studies conversely report that PTC can be characterized by a low level of Notch signaling (Xiao et al. 2009; Yu et al. 2016). Ultimately, the controversial findings regarding Notch signaling in PTC highlight the complexity of the pathway and the need for continued investigation.

In thyroid cancer subtypes that can be characterized as less differentiated, it appears that Notch signaling is decreased. This observation is supported by evidence that demonstrates lower levels of Notch signaling in FTC, MTC, and ATC in addition to a significant association found between low levels of Notch receptors and more aggressive cases of thyroid cancer (Ferretti et al. 2008; Xiao et al. 2009; Yu et al. 2016; Somnay et al. 2017). ATC, the most poorly differentiated subtype, has the lowest expression of the Notch1 receptor when compared to more differentiated thyroid cancers and normal thyroid cells (Ferretti et al. 2008; Patel et al. 2014; Yu et al. 2013b; Somnay et al. 2017; Piana et al. 2019).

The majority of literature that describes Notch signaling in thyroid cancer focuses on the Notch1 isoform, followed by the Notch3 isoform. There is minimal investigation into the other two known Notch receptors (Notch2 and Notch4). One study on MTC reported that the natural compound resveratrol was able to increase Notch signaling through an induction of Notch2, which is consis-

tent with the study that reported a similar effect on Notch1 (Truong et al. 2011; Yu et al. 2013b). The individual and synergistic roles of each Notch receptor (Notch1–4) have yet to be understood in thyroid cancer. A deeper understanding of each receptor and the interplay between them could greatly advance what is known about Notch signaling and potentially lead to new strategies for modulating the pathway.

The paradoxical balance between oncogenic Notch and tumor-suppressive Notch impacts the modulation strategy. Various natural compounds have been identified that induce Notch signaling in thyroid cancer, thus exploiting a suspected anticancer effect by high levels of Notch (Rashid et al. 2018; Tesfazghi et al. 2013; Yu et al. 2013b; Patel et al. 2014; Yu et al. 2013a; Damaskos et al. 2016; Adler et al. 2010; Spartalis et al. 2019; Jang et al. 2015). Such compounds have demonstrated promising preclinical data, but have yet to be explored in a clinical setting. Compounds that have been tested clinically to increase Notch signaling in thyroid cancer, primarily HDAC inhibitors, have resulted in disappointing findings. The use of HDAC inhibitors as a monotherapy for the treatment of thyroid cancer has largely been ineffective. The inconsistency between promising preclinical results and disappointing clinical results could be attributed to additional mechanisms of action by which HDAC inhibitors effect thyroid cancer cells that have yet to be understood. Further understanding how Notch signaling functions in thyroid cancer could lead to advancements in the design of clinical trials that target the pathway. Notch pathway has been cited as a potential prognostic marker in patients with thyroid cancer, although this would also require a more definite role of Notch signaling in these tumors (Jung et al. 2017).

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The Relevance of Notch Signaling in Cancer Progression

11

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Abstract

The Notch signaling pathway controls normal embryonic development and tissue homeostasis of many cell types. It regulates cell proliferation, fate, differentiation, and cell death by short-range signaling between nearby cells that come in contact. The Notch pathway has also been critically involved in the pathobiology of a variety of malignancies, regulating cancer initiation and development, as well as early stages of cancer progression, by adjusting conserved cellular programs. Fibroblasts, an essential for tumor growth component of stroma, have also been affected by Notch regulation. Sequencing Notch gene mutations have been identified in a number of human tumors, revealing information on the progression of specific cancer types, such as ovarian cancer and melanoma, immune-associated tumors such as myeloid neoplasms, but especially in lymphocytic leukemia. Activation of the Notch can be either oncogenic or it may contain growth-suppressive functions, acting as a tumor suppressor in other hematopoietic cells, hepatocytes, skin, and pancreatic epithelium.

Keywords

Cancer · Cancer progression · Notch · Oncogene · Tumor suppressor · JAG · Delta-like ligand

Introduction

The capability of tumor cells spreading to distant and neighboring organs reveals the complexity of cancer. Vascular network growth is essential for the metastatic growth of cancer tissues. Tumor cells are able to penetrate both the lymphatic and the blood vessels, circulating the intravascular stream, proliferating at different tissues, and creating the appropriate risks for metastasis (Folkman and Judah 1971). Especially angiogenesis, but also lymphangiogenesis referring to the new blood and lympatic vessel formation respectively, are both vital for tumor growth and progression. The two have an essential role in forming a vascular network that supplies nutrients, immune cells, and oxygen, as well as removing waste products (Folkman 1974). Factors leading to both lymphangiogenesis and

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angiogenesis are a primary concern, mainly in vascularization and neoplastic fields. During the latest decades, Notch signaling pathway has proven to have a key role in vessel formation and therefore cancer development, raising questions about unexplored therapeutic pathways of altering Notch expression.

Angiogenesis in Cancer

Metastasis and tumor growth are dependent on the lymphangiogenesis and especially on angiogenesis processes, which are often induced by chemical signals originating from cancer cells during the rapid growth phase (Folkman and Judah 1971). A comparison of the cancer cells' behavior infused into distinct areas of the same organ revealed the need of blood supply for tumors. Among the parts was an iris, which had the blood circulation, while the anterior chamber was used as the region with no blood support. Without the blood circulation, tumor was found out to grow to a maximum diameter of 1–2 mm^3, but was able to further develop past 2 mm^3, when put in an area with circulation support (Muthukkaruppan et al. 1982). The absence of vascular support leads cancer cells to inhibit tumor development (Holmgren et al. 1995). For that reason, angiogenesis is a factor crucial in cancer progression (Fig. 11.1). Neovascularization for tumor support and progression involves four main stages. The first stage is the local injury of the basement membrane in tissues resulting in instant hypoxia and destruction. Secondly, endothelial cells activation by migrating angiogenic factors. The third part involves the proliferation and stabilization of the endothelial cells. In the last part, angiogenic factors promotes vascularization. Denekamp affirmed that vascular endothelial cell division occurs just in every 1000 days' average (Denekamp 1993).

Angiogenesis Via Notch Stimulation

Angiogenesis stimulation occurs if the tumor tissues are in the requirement of both nutrients and oxygen. Activator and inhibitor chemicals are critical for the angiogenesis regulation. Nonetheless, the activity upregulation of angiogenic factors is never enough for neoplasm angiogenesis, while, downregulation of noninhibitors and regulators of vessel growth are also essential (Dameron et al. 1994).

Currently, the idea of specific pathways having a significant role in tumor angiogenesis and vascular function is received widely. The cell-to-cell signaling known as the Notch signaling pathway is found out to play a vital part in tumor angiogenesis, mostly via the Delta ligand 4 (DII4), one specific Notch receptor ligand (Dufraine et al. 2008; Hoey et al. 2009; Gurney and Hoey 2011; Ribatti and Crivellato 2012). A trigger in the Notch pathway results in the initiation of the sequential receptor proteolytic cleavages; ligand proteins always stick to extracellular domains. Consequently, the release cleavages and intracellular domain gets induced, thereby entering the cell nucleus and modify the expression of the gene. Every cell accompanies a diverted biochemical trail (Hao et al. 2010).

The Notch signaling pathway enhances the mediation of the cell fate. Additionally, it is a crucial parameter in each local cell-to-cell system of communication. It also coordinates a pathway of signaling that requires regulation of the mechanisms of the gene responsible for the control of several procedures in cell differentiation. Moreover, it is entailed in the varied processes of organogenesis, embryogenesis, and renewal of both tissues and cells (Klinakis et al. 2006; Schepers et al. 2002). NECD–NTMIC are single-pass Notch cellular receptors transmembrane proteins, which include an extracellular, an intracellular domain, and transmembrane. Four receptors are always found in mammals, including receptors NOTCH 1, NOTCH 2, NOTCH 3, and NOTCH 4. Within the cells that receive signals, there are endoplasmic reticulum and the Golgi bodies, where the processing of the receptors takes place.

The generation of stabilized calcium heterodimer of NCED, which is not covalently in the attachment to TM-NCID inserted in the membrane, is done by the glycosylation and cleavage. As stated by one model, after being cleaved away from the TM-NICD domain by TACE, NECD enters the endosomal system of the signal-send

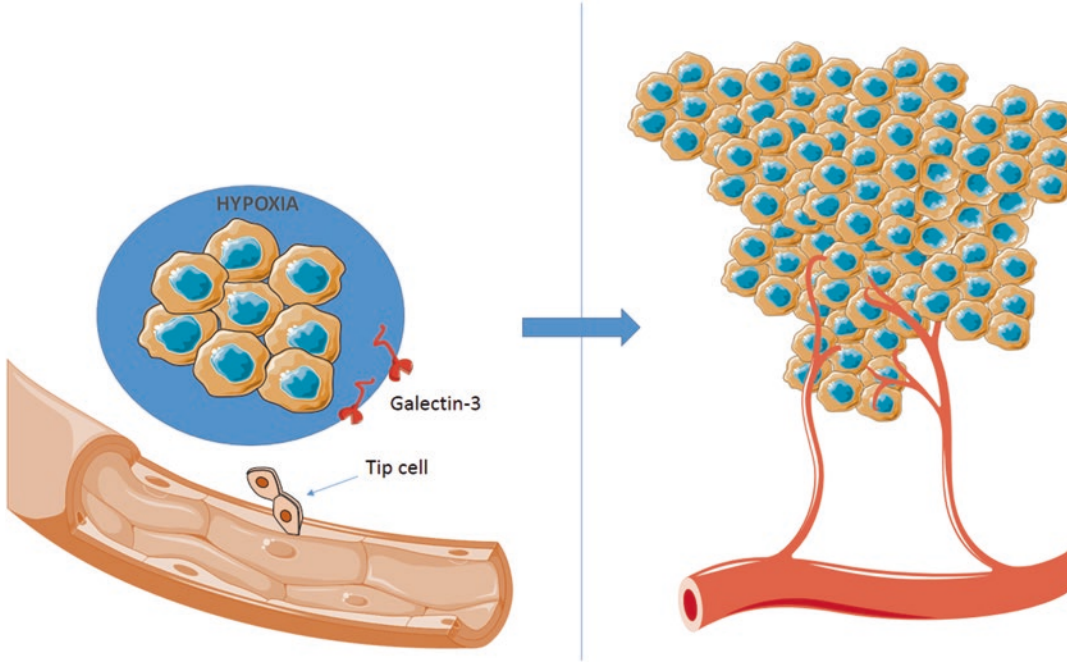

Fig. 11.1 Neovascularization due to exposed to hypoxic – environment cancer cells. In absence of blood support, tumors are unable to grow beyond 1–2 mm^3

Table 11.1 Notch signaling pathways target genes

Role	Target gene
Apoptosis	NFKB1, CDKN1A, CFLAR, IL2RA
Cell cycle regulators	CCND1, P21, P27, IL2RA
Cell proliferation	P21, P27, ERBB2, FOSL1, IL2RA
Cell differentiation	DTX1, HES6, PPARG
Neurogenesis	HES1, HEY1, HEY2
Transcription	NFKB1, NR4A2, PPARG, STAT6, DTX1, HES1, HES6, HEY1, HEY2, FOS, FOSL1
Unspecified	CD44, CHUK, PTCRA, LOR, MAP2K7, PDPK1, MGC61598, HES5, IFNG, IL 17B, IVL, KRT1, KRT10, KRT14, KRT5, LOR

cell. This results in the recycling of the NECD part in the cell plasma, and the γ secretase frees the NICD from TM in the receiving signal cell. NICD part gets into the nucleus, with CSL transcription factor complex activation permits the translocation nucleus, thus leading to the actuation of notch canonical target genes (Fig. 11.2 and Table 11.1). Examples of Notch agonists are jagged and Delta-like ligand proteins (Karamboulas and Ailles 2013; Wang et al. 2015). Delta-like protein examples include Delta mammalian homologs, which take part as ligands for Notch 4, 3, and 1 pathway receptors and the Drosophila protein. Human beings the D114 encoding is by the D114 gene. Even though many of the Notch ligands and receptors express various types of cells, Jag 1 and D114 disclose an extremely discriminating pattern of expression within the vascular endothelium and majorly in actively growing vessels and already formed arteries. Hence they reveal a significant role in the promotion of angiogenesis (Shutter et al. 2000) (Fig. 11.2).

Notch Ligands' Role in Angiogenesis

The vascular structure of the tumor is untypical and exhibits deviant functional and morphological characteristics. Diligently the tumor recruits the blood vessels by the inducement of sprouting

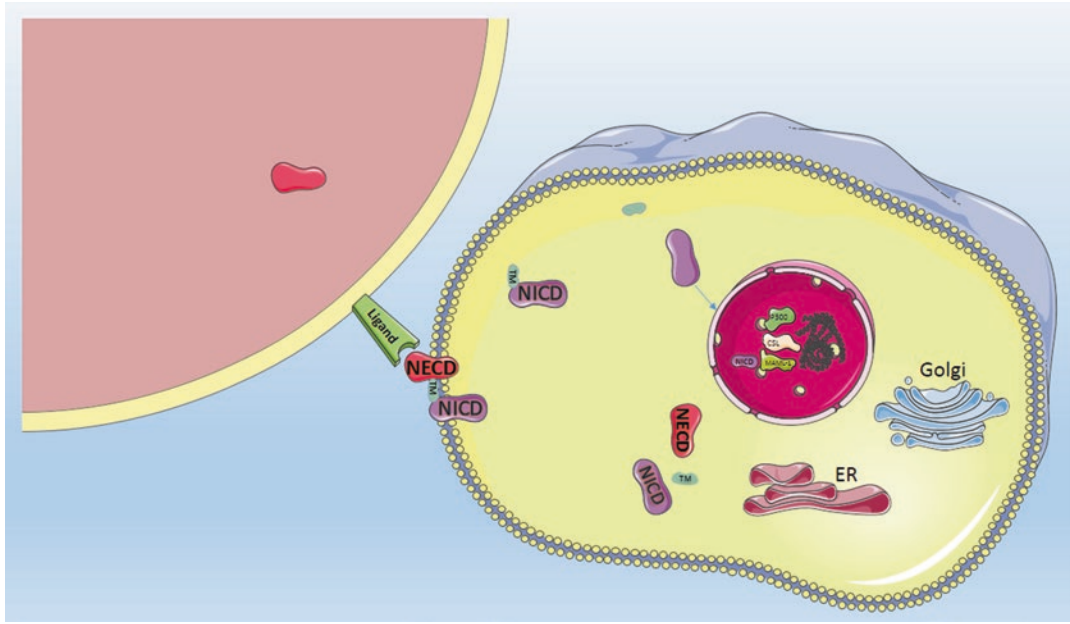

Fig. 11.2 The notch pathway. Construction of the receptor in the endoplasmic reticulum and Golgi is followed by cleavage of NECD from TM-NICD with the converting enzyme TACE. The processed NECD is endosome-transported in the signal-sending cell plasma membrane where it is recycled. γ-secretase releases NICD from TM in the signal-receiving cell and the NICD part enters nucleus and with the activation of CSL transcription factor complex allows nuclear translocation resulting in activation of the canonical notch target genes

existing blood vessels, which in turn distributes nutrients to the tumor cells. Data suggest that Notch is the primary emergence angiogenesis regulator (Hellström et al. 2007). The regulator control is by a firmly controlled balance among stalk cells and endothelial tip. Cell tip differentiation is in response to factors of pro-angiogenic to develop vasculature. Notch signaling and especially Dl14 dominates the endothelial tip cell emergence. Notch-conciliated inhibition of VEGFR2 supports the phenotype of the stalk in avoidance of hyper-sprouting, thus dominating the vasculature architecture. The mechanisms of Notch regulation of sprouting are often not specific to settings of cancer (Benedito and Hellström 2013).

Notch ligands modify the tumor compartments through the activation of the Notch signaling in malignant tumor cells. Various observations indicate that endothelium-expressed Notch ligands can induce Notch signaling in nearing tumor cells. Particularly, aiming mouse D114 in the model of xenograft decreases the activity of Notch in malignant tumor cells (Kuhnert et al. 2015). In glioblastoma, it is manifested that the operation of Notch in cancer cells is more significant in the closeness of endothelium cells (Lu et al. 2013; Zhu et al. 2011). The demonstration of this is prevalent in diverse cancer kinds and is likely to include various Notch glands and receptors. For instance, D114 conveyed by endothelium cells activates Notch 3 in the cells of T-ALL and permits the escape of dormancy (Indraccolo et al. 2010). Notch activation in tumor cells by nearing blood vessels is additionally perceived to increase the migration of transendothelial, thus resulting in spread (Sonoshita et al. 2011). Moreover, it is indicated that Jag1 expression by endothelial cells leads to activation of the signaling Notch in the pericyte precursor local cells to help induce differentiation pericyte (Patenaude et al. 2015). Endothelial cells–expressed ligands are also responsible for the control of cell traits cancer stem. Therefore, the control of most tumor vasculature aspects is made by the Notch signaling, which also dominates survival and differen-

tiation of endothelium, by the inherent mechanisms of heterotypic relations with cancer. Notch-conciliated shaping regulates the resistance of tumors. The resistance infiltrate comprises of immune cells which takes parts in cancer cells and is acknowledged as the primary tumor progression regulator.

Notch receptors can work as cell autonomous tumor suppressors, cell autonomous oncoproteins, or microenvironment-dependent oncoproteins in distinctive cellular contexts.

Nonetheless, the mechanism regulation is based on the tumor, and questions arise considering the implications of Notch on therapeutic pathways. Since Jag 1 and D114 possess different roles in the control of angiogenesis development, the stability among Jag1 and D114 has a significant effect on the tumor blood vessel architecture (Benedito et al. 2009). The mathematical modeling indicates that substantial levels of Jag1 are likely to result in chaotic and imperfective perfused angiogenesis by the destabilization of the stalk or tip phenotype (Boareto et al. 2015). High expression of endothelial cells' Jag 1 causes a rise in tumor blood vessel network, while Jag1 function loss in endothelial cells results in reduced tumor growth and vasculature (Pedrosa et al. 2016). In the regulation of the tip ratio, Notch again implies the regulation escape of metastasizing from dormancy the cells causing cancer, as tip cells have an association with this procedure (Ghajar et al. 2013).

Notch Pathway Regulating the Immune System–Associated Tumors

Notch tumor inducement that activates endothelium may also result in endothelial senescence in myeloid and tumor cells, which prompts inflammation and an increase in the spread (Wieland et al. 2017). Therefore, the expression of Jag1 by the tumor cells is beneficial competent in vasculature control. The variations of solid tumors reveal the evidence of CD8+ infiltration of the T-cell, which has a density of powerful prognostic effects in several solid tumors (Fridman et al. 2017). The infiltration has an association with the type 1 transcriptional interferon signature, illustrating the inherent immunity activation. This anticancer infiltrate effectiveness is lessened by controlled physiological mechanisms, namely, myeloid-derived suppressor cells and the recruitment of regulatory T-cells. The two form an immunosuppressive domain in cancer cells (Gajewski et al. 2013). Notch signaling also affects the development of B and T lymphocytes, and especially the regulation of B subset lymphocytes of the marginal zones in addition to function and differentiation of innate lymphoid and dendritic cells (Radtke et al. 2013).

Notch signaling is also essential for the control of CD8+ cytotoxic T-helper-cell activation, the significant actor of the antitumor. The naïve CD8+ T-cell activation needs the binding D111 to Notch2 or Notch1 for interferon and granzyme B expression (Cho et al. 2009). Alongside this, Notch2 is evidently to be a requirement for an antitumor CTL reaction (Sugimoto et al. 2010). It is depicted that the restoration of the Dll1–Notch pathway in the domain of blood cell establishment restores the T-cell role (Huang et al. 2011). Furthermore, it supports the Notch signaling function in supporting antitumor T lymphocyte undertaking, treatment employing multivalent Dll-1–induced decrease of tumor spread through the elicitation of lymphocyte and differentiating T and the enhancement of cytotoxicity of antigen-specificity (Biktasova et al. 2015). The facts indicate the positive function of the Notch signaling in the regulation of CD8+ T-cells antitumor activity. Investigations are ongoing in examining how the antitumor activity regulation happens once the lymphocytes get into the tumor through the expression of the ligands in the cancer cells.

Nonetheless, the dependent context role of Notch needs to be made clear because the prevention of Notch signaling in CD8+ T-cells from patients diagnosed with colon cancer leads to rising in their cytotoxic mechanism by reducing PD-1 expression (Yu et al. 2018). Additionally, Notch signaling is significant in regulating the various constituents in the immunosuppressive environment. The notch is crucial in differentiating tumor-associated macrophages (TAM).

The macrophages exhibit a transcriptomic signature, which is related to the Notch signaling pathway (Liu et al. 2017). The deletion of CSL in monocyte origin blocks both the tumor-associated macrophages and the related immunosuppressive roles, while differentiation of TAM may additionally be modified by cells immune to therapy (Franklin et al. 2014).

The cells mostly show Jag1 and enhanced TAM markers in cultured macrophages. The rise is dumped when the cells are served with inhibitors of g-secretase (Franklin et al. 2014). Some research illustrates that Notch signaling has involvement in expanding the phenotype of myocardial infarction (MI) macrophage. Data support that macrophages' inadequacy in Notch signaling has a reduced antigen appearance mechanism (Wang et al. 2010). Besides, the forced Notch activation in macrophages by the expression of NIICD suppress the spread of tumor by revoking TAM functions (Zhao et al. 2016). Thus, macrophage's polarization depending on the intrinsic Notch signaling action of macrophages, adjusted by interacting with alternative cell types that express the notch ligands in cancer cells.

Key characteristics of the third mutational pattern include frame shift, disruptive nonsense, or point substitutions observable at the N-terminal portions of the Notch receptors. In anticipation, each of these is anticipated to illustrate a loss of Notch function. A share of these mutations results in a failure to produce protein for the Notch protein. However, there is an expression of outstanding negative decoy receptor which contain either deleted or disabled intracellular domains that are motivated by occasional truncations or point mutations. In squamous cell carcinomas of the skin, there is a prevalence of loss-of-function mutations occurring in the Notch receptors, head and neck, esophagus, and lung. They may similarly be seen in lung cancers with small-cell, low-grade gliomas and urothelial carcinomas (Wang et al. 2011; Agrawal et al. 2011; Stransky et al. 2011; Agrawal et al. 2012; George et al. 2015; Rampias et al. 2014).

In squamous cancers, the realization of Notch mutations that were disruptive was presaged by experiments proving that Notch1 deletion significantly raises the incidence of skin tumors in studies of mice exposed to carcinogens, where the dominant negative MAML1 potently induces the cutaneous squamous cell carcinoma (Nicolas et al. 2003; Proweller et al. 2006). Deletions of RBPJ were identified in tumors where the Notch has a distinguished role of suppressing the tumor. Such a tumor includes the squamous cell carcinoma. It suggests that there is a complex relationship between altered RBPJ gene dosage and carcinogenesis and, as a result, differences may be seen depending on tumor type. In some minor cases of cancer, there have been reports of somatic mutations which incorporate elements of the Notch that signal a pathway different from the Notch receptors. However, a potential exception to this rule is traceable to a recent study. The study reports that a frequency in RBPJ copy a loss and reduction in RBPJ protein expression in a sizable minority of different types of carcinoma – especially those in breast cancers. A proposal for the reduction in dosage of RBPJ gene in cancers whose Notch has an oncogenic role (breast cancer as an example), and drives the derepression of target genes of the oncogenic Notch is made. Perhaps, this is proposed due to a failure in RBPJ to recruit corepressors for the genomic regulatory elements (Kulic et al. 2015). In other studies, MAML2 has been shown to have frequently engaged in translocations within mucoepidermoid carcinoma (Tonon et al. 2003). Despite this, the CRTC1–MAML2 fusion gene which is developed appears not to alter the signaling of the Notch.

Notch-Mediated Relation with and Activation of Fibroblasts

Of importance to cancer environment is the associated cancer fibroblasts. Cancer-associated fibroblasts (CAFs) are seen to take part in disease progression, initiation, and metastasis (Gascard and Tlsty 2016; Meurette and Mehlen 2018). Activation expression markers distinguish the cancer fibroblasts, namely, fibroblast activation, smooth muscle actin, and many produced factors involved in remodeling of the establishment of

immune infiltrate and modification of extracellular matrix. The significance of Notch signaling in the control of fibroblast activation is currently established within the tumor. Notch signaling in the human tumors is reduced in stromal cells adjoining precancerous lesions, keratinocytes, and the reduction of Community Service Locator in mesenchymal cells in mice is enough to prompt multifocal keratinocytes cancers (Hu et al. 2012). This indicates a casual impact of signaling (Meurette and Mehlen 2018).

Notch canonical is related to the differentiation of CAFs (Procopio et al. 2015). Notch 1 is the primary controller of fibroblasts senescence as it might be a portion of its function in stromal activation regulation in the course of the cancer development procedure. The impact of the loss of Notch1 can orient the senescence-associated phenotype in the direction of a proinflammatory and can thus take part in the initiation of the tumor. Notch activity in fibroblasts is additionally crucial after the forfeiture of the cancer-suppressive undertaking of Notch in the skin epithelial section. The reduction of Notch 1 in the chamber of epithelial cells connect to noncell autonomous modification in the stroma (Demehri et al. 2009). In Notch pathway, colon cancer is evidenced to conciliate activation of bone marrow-generated stromal cells to fibroblast activation. The impact of Notch in the stroma can rely on the steps of tumor spread (Peng et al. 2014). As a fact, the Jag1 manifestation by malignant tumor cells in prostate cancer is evidenced to enhance a rise in activated fibroblasts that expresses a-SMA and developing a stroma that is active with intensified collagen and tenascin (Su et al. 2017). In such situations, the Notch activation, despite its loss, is involved in the fibroblast activation. CAFs, besides, can induce Notch signaling in the cells of cancer. For instance, CAFs identified to prompt the activation of Notch in the breast cancer cells through the secretion paracrine IL-6 (Studebaker et al. 2008). CAFs are responsible for promoting malignant tumor stem cell phenotype in breast cancer; through CCL2 secretion (Tsuyada et al. 2012). Notch3 activation by CAFs is also linked with the spread in cancer stem cells in liver carcinoma (Liu et al. 2018). The Notch-conciliated association among the mesenchymal compartments and cancer cells also entails chemotherapy resistance. Especially in breast cancer, fibroblasts express Notch ligand Jag1, which can react with Notch3 and balance resistance (Boelens et al. 2014).

The Notch 3 induction is prevailed upon by the stromal cells–generated exosomes, which trigger STAT-1-dependent antiviral signaling in the cells of cancer (Boelens et al. 2014). The interchange among the activated stroma and cancer cells is, therefore, in portion-controlled by the Notch signaling. As a fact, the modification of the Notch signaling in the mesenchyme or epithelial compartments has an intense effect on the other partitions. The Notch-dependent interchange among the malignant tumor Stem Cell Pool Notch signaling is a determinant significant of the stem cell support in the various distinct cancerous tumors (Takebe et al. 2015). Stem cells present in a niche dependent-context in which constituents of the tumor cells engage in a crucial function. In both co-culture and in vitro, endothelial and glioblastoma cell experiments indicate that the existence of endothelial cells gives rise to cancer stem cells population (Hovinga et al. 2010).

The scrutiny is confirmed by the analysis in tumors indicating that Notch signaling is induced in the cancer cells by activators stimulated by the vascular locality (Zhu et al. 2011; Bayin et al. 2017). Differently, Jag1 contributes a significant function in the relation of cancer cells with the local area. In B-cell lymphomas, the vascular space is involved in offering Jag1 to cancer stem cells, which trigger Hey2 via Notch2 to strengthen resistance and aggressive phenotype (Cao et al. 2014). In malignant colon tumors, endothelial-expressed Jag1 promotes cancer stem cell replenishment through the production of soluble Jag1 (Lu et al. 2013). Jag1 is responsible for the proliferation of stem cells and the formation of the stem cell niche in an antigen-presenting cell-deficient model of intestinal tumors (Nakata et al. 2017). In breast cancer, CCL2, which are derived from fibroblasts, facilitates Notch1 to maintain the phenotype of stem cells (Tsuyada et al. 2012). Therefore, the primary Notch ligand provided is

Jag1 by stroma, which supports the stem cell phenotype. The ability of Jag1 to trigger a specific transcriptional program is the reason for the particular function of Jag1. In the neck and head cancer cells, Jag1 triggers Kfl4 expression, which leads to stem cell phenotype and the resistance to treatment (Chen et al. 2014). Notch signaling is a significant pathway in mediating the relationship between the niche of the various cancer stem cells.

Pathways Regulating Notch in Cancer

The collaborations which involve signing amid distinct sections of tumors defined can be enabled by crosstalk with other pathways that influence the presence of Notch ligands and their receptors. In B-cell lymphoma, cancer cells secrete FGF4, which triggers Jag1 presentation in the adjacent endothelial cells that induce Notch2 in lymphoma cells (Cao et al. 2014). Notch signaling is regulated by the IL-6/STAT3 pathway in cancer environment through relationships amid various sections. Data support that the initial medical evidence of the control of Notch signaling is through IL-6 in which the induction of Jag1 autocrine production by tumor cells in breast tumors (Sanguinetti et al. 2015).

Wnt signaling targets Jag1 in colon and breast cancers (Ayyanan et al. 2006; Rodilla et al. 2009). The forfeiture of Hippo pathways triggers hepatic disease in a manner dependent on Jag1 (Kim et al. 2017). In cancer cells, the IL-6/STAT way assists in the control of Notch signaling via the relations among the distinct components. Moreover, crosstalk between IL-6/STAT3 and Notch occurs between cancer cells and CAFs in hepatocellular carcinoma (Xiong et al. 2018). The crosstalks were among cancer cells and mesenchymal stems in the cancer of the colon (Lin et al. 2013). The Notch receptor signaling in myeloma regulates the secretion of IL-6, which reshapes the bone marrow environment (Colombo et al. 2016). Additionally, Notch receptors' expression is controlled by the crosstalk in tumor cells. For instance, in the cancer of the breast, Notch 3 inducements in malignant stem cells are by the RIG-1/STAT1 pathway that has its activation by the stromal cell-expressed Jag1, which may even cause resistance to therapy (Boelens et al. 2014).

Transforming growth factor-β (TGF-β)pathway is another essential way included in the crosstalk in tumors with Notch. TGF-β upregulation in the stroma of prostate cancer cells results in the formulation of a stroma that is reactive (Su et al. 2017). Myeloid cell-dependent Notch activation in cancer cells cause TGF-b enhancement that lead to pSMAD2/3 pathway activation (Ohnuki et al. 2014).

Notch as a Tumor Suppressor

Even though the activation of the Notch can be oncogenic (particularly at greater levels as conferred by ICN1 expression), mounting evidence suggests that components with a similar pathway may contain growth-suppressive functions in skin, hepatocytes, hematopoietic cells, and pancreatic epithelium (Lobry et al. 2011).

The suprabasal cells in the skin were found to largely labor the Notch receptor and ligand expression. Alternatively, in vitro data illustrated that Notch activation induces differentiation and arrest of the cell cycle. Deletion of NOTCH1 in the skin conditionally culminated in a staggering rise of the epidermal layer at the base. In line with a function to suppress tumor for the Notch in the skin, the loss of purpose for NOTCH1 led to spontaneous basal cell carcinomas, which emerged in older mice as well as the sensitization of skin carcinogenesis that was induced chemically (Stransky et al. 2011; Lowell et al. 2000; Rangarajan et al. 2001; Nguyen et al. 2006). In addition, Notch plays the role of tumor suppressor in the skin by suppressing the Wnt and Sonic–hedgehog pathways. The tumorigenic effect associated to the deletion of Notch1 comes from a noncell autonomous defect in the skin barrier's integrity (Demehri and Kopan 2009). Mechanistically, the inhibition of tumor in the skin may consist of feedback with the microenvironment as well as crosstalk between Notch and other pathways for signaling.

A suppressor role for the novel tumor by the Notch signaling in hepatocellular carcinoma (HCC) was recently suggested. The study included a mouse model of HCC cancer, by ridding the retinoblastoma protein (RB) and its two related family members p107 and p130 in liver cells, to fully understand the initiation and progression of HCC. For the triple KO (TKO) mice utilized for the study, liver cancer with histological and molecular features commonly seen in human cancer was developed. For the model used, there was an expansion of the stem/progenitor compartment in the liver due to a lack of activation of the RB pathway. It is suggested that these adult progenitor cells are responsible for activating HCC tumor cells after RB inactivation. In the TKO mice, both RB pathways were upregulated, in support and agreement with previous findings suggesting that hyperactivation of E2F and Myc signals are sufficient to induce HCC (Lobry et al. 2011). Using transcriptome profiling and gene set enrichment analysis, an upregulation in the Notch pathway in the TKO mice was noted, proving the oncogenic role for Notch signaling in HCC development (Viatour et al. 2011). Moreover, the Notch signaling inhibition in the TKO mice using DAPT, a potent γ-secretase inhibitor, resulted in an accelerated development of HCC. Enforced activation of Notch signaling using ICN1 led to cell cycle arrest and apoptosis in primary HCC cells isolated from TKO mice, in addition to the human HCC cell lines. To further address the importance of these observations clinically, the Notch activation status in a patient cohort was also examined. The results support that the expression of Notch-related genes such as HES1 was higher among patients with better survival rates supporting the role of Notch signaling in HCC as a tumor suppressor (Lobry et al. 2011).

The conditional Notch loss-of-function via the elimination of Nicastrin (NCSTN), a vital part of the γ-secretase complex, or compound deletion of NOTCH1 and NOTCH2, leads to a myeloproliferative syndrome with similar features to the chronic myelomonocytic leukemia human disease (Klinakis et al. 2011). An analysis on the whole transcriptome established that Notch signaling prevented an early differentiation program of the monocytic/granulocytic in an early multipotential progenitor, mediated through the direct repression of the PU1 and C/EBPα promoters by HES1. It was revealed that ∼12% of CMML patients had inactivating mutations in NCSTN, MAML1, APH1A, or NOTCH2 through the sequencing of Notch pathway genes. The uniqueness of these mutations was only traceable to CMML and lacked in other myeloproliferative disorders like myelofibrosis and Polycythemia vera. Contrary to the function of Notch in epithelial cells and HCC to suppress tumors, there is a suggestion among these studies that Notch signaling may impede the proliferation and transformation of myeloid cells that may lack control during hematopoietic development.

Furthermore, mutations impacting Notch receptors were identified by two recent studies which focused on head and neck squamous cell carcinoma (HNSCC). Among 21/120 patients were identified 28 different NOTCH1 mutations (17.5%). Of these, 11 were nonsense or insertion/deletions with the potential to cause a functioning loss and as such, countenancing the function of Notch in HNSCC to suppress tumors. The rest (17 in total) were missense mutations which mostly were found in the extracellular EGF-like repeats that are crucial in the interaction of receptor–ligand. In 11% of patients analyzed in a study, NOTCH1 mutations were identified whereas an additional 11% of patients harbored the NOTCH2 or NOTCH3 mutations (Stransky et al. 2011). The missense, nonsense, or insertion/deletions mutations which target the extracellular domain of the Notch receptors were all identified. They are therefore predicted being responsible for the loss-of-function while implicating NOTCH1 as tumor suppressor in HNSCC. In addition, evidence that the Notch in B-cell malignancies suppressed growth and induced apoptosis were revealed. This solidified the view that the Notch could also suppress a tumor in hematopoietic cells and in neuroblastoma (Zage et al. 2012; Zweidler-McKay et al. 2005). However, even though most of the studies used in vivo samples, further investigation could clarify the exact role of Notch signaling pathway

in cancer formation, and its role as oncogene, or tumor suppressor gene.

Conclusion

The Notch pathway is involved in cell–cell communication and plays a critical role in cell fate determination while there are many developmental cell fate decisions that are controlled by the Notch pathway through the regulation of genes that engage in differentiation and proliferation. The development of different types of cancers are highly influenced by the deregulated Notch signaling due to the Notch genes depending on tissue type and able to act either as oncogenes or tumor suppressor genes. Notch signals on cellular transformation can at times have complex effects. These are a clear representation of the protean effects of this signaling pathway on cells and tissues that develop normally. Signaling components such as Notch receptors and ligands have patterns of expression that overlap dynamically. This raises the possibility of this pathway being subject to extensive fine-tuning to guarantee that the Notch signals' timing and strength is appropriate in context. Commonly, cancers are identical to cells which correspond the normal developmental stages. Hence, the fact that there is a wide expression of Notch signaling molecules in different neoplasms is not appalling. Differentiating stage-appropriate expression of Notch signaling components from aberrant expression that is pathophysiologic is however the daunting task.

Currently, the trend in cancer therapy is to replenish systemic chemotherapy with target-specific factors, including chemicals like all-trans retinoic acid and imatinib mesylate (STI571). Many differentiation processes are affected by the wide expression of the Notch. Hence, the toxicities attributed to the targeting of this pathway may currently not be valid. This primarily gives room to the centralized focus on the downstream mediators of Notch signaling as opposed to components of the central signaling axis. Enhancing the comprehension of Notch signaling in normal development in addition to malignant transformation may result in novel cancer therapeutics.

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Notch Signaling and the Breast Cancer Microenvironment

12

Qiang Shen and Michael Reedijk

Abstract

Notch promotes breast cancer progression through tumor initiating cell maintenance, tumor cell fate specification, proliferation, survival, and motility. In addition, Notch is recognized as a decisive mechanism in regulating various juxtacrine and paracrine communications in the tumor microenvironment (TME). In this chapter, we review recent studies on stress-mediated Notch activation within the TME and sequelae such as angiogenesis, extracellular matrix remodeling, changes in the innate and adaptive immunophenotype, and therapeutic perspectives.

Keywords

Notch · JAG · DLL · γ-secretase · RBPJκ · Breast cancer · Triple negative · Basal-like · Tumor microenvironment · Angiogenesis · Urokinase-type plasminogen activator · Extracellular matrix · TRB3 · USP9x · Cellular stress · TGF-β · IL1β · CCL2 · Tumor-associated macrophage · CD8+ T-cell · Immunophenotype · Cancer-associated fibroblast · PD-1 · Immune checkpoint blockade

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Introduction

Breast cancer is the most frequently diagnosed malignancy and the second leading cause of cancer death in North American women (Ferlay et al. 2015). As a heterogeneous disease with various patterns of gene expression and prognosis (Sorlie et al. 2001), breast cancer can be grouped into three major clinical subtypes (Fig. 12.1): the estrogen receptor (ER) and/or progesterone receptor (PR) expressing luminal A/B subtype; the human epidermal growth factor receptor 2 (HER2)–amplified subtype; and a third subtype, triple-negative breast cancer (TNBC; lacking ER, PR, or HER2) that overlaps significantly with the basal-like breast cancer (BLBC) molecular subtype. TNBC/BLBC comprises 15% of all breast cancers, primarily affecting young women, people of African or Hispanic ancestry, and those with BRCA1 mutations. With advances in understanding breast cancer at the molecular level, and the development of targeted therapies for ER-positive and HER2-positive subtypes, mortality in breast cancer has decreased significantly. However, therapeutic challenges still persist for the TNBC/BLBC subtype, which is aggressive, has poor prognosis, and for which known effective therapeutic targets are lacking. In the past decade, a concerted effort has been made toward identifying molecular targets for this elusive subtype, and some exciting progress has been made, including the discovery of the Notch signaling pathway as a potential therapeutic target (McCann

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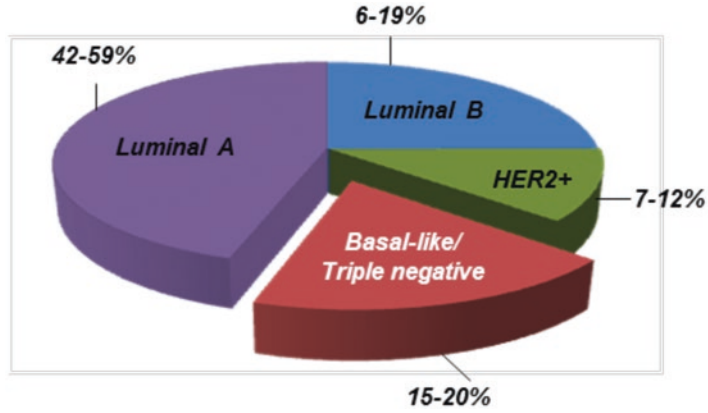

Fig. 12.1 Clinical subtypes of breast cancer

et al. 2019; Vidula and Bardia 2017; Locatelli and Curigliano 2017; Mollen et al. 2018; Argyle and Kitamura 2018; Izrailit and Reedijk 2012).

Notch normally regulates mammary stem cell maintenance and progenitor cell fate and is indispensable for normal mammary gland development. Not surprisingly, aberrant activation of Notch in the mammary gland promotes mammary tumorigenesis and malignant progression (Callahan and Raafat 2001), particularly in breast cancers of the TNBC/BLBC subtype (Izrailit and Reedijk 2012; Reedijk et al. 2005, 2008). Recent findings suggest that Notch promotes tumor progression in part, by shaping the tumor microenvironment (TME), specifically by determining the tumor immunophenotype (Shen et al. 2017; Boelens et al. 2014; Studebaker et al. 2008). The TME, where tumor cells dynamically interact with resident and recruited "nonmalignant" cells is crucial to malignant progression and metastasis (Balkwill et al. 2012). In the breast TME, the "nonmalignant" cell components include immune cells of both the innate and adaptive systems such as tumor-associated macrophages (TAMs) and tumor-infiltrating lymphocytes (TILs), respectively (Binnewies et al. 2018; Guerriero 2018). This chapter reviews evidence that Notch regulates tumor angiogenesis and extracellular matrix remodeling, as well as more recent progress toward understanding how Notch shapes the immune TME. These findings provide a rationale for the development of immunotherapies that target Notch and the Notch interactome.

Overview of Notch Signaling in Breast Tumorigenesis

The Notch signaling pathway plays an essential role in intercellular communication and tissue patterning (Nichols et al. 2007). Upon binding the ligands Delta-like (DLL1, DLL3, and DLL4) or Jagged (JAG1 and JAG2) on neighboring cells, Notch receptors (NOTCH1–4) undergo a series of proteolytic cleavages, including a presenilin–protease (γ-secretase)–mediated cleavage that releases the active cytoplasmic domain fragment, Notch intracellular domain (N^{IC}), from the plasma membrane (Fig. 12.2). N^{IC} then translocates to the nucleus where it engages the DNA-binding protein CSL (CBF-1/suppressor or hairless/LAG-1), also known as RBPJκ, resulting in replacement of a multiprotein corepressor complex with a coactivator complex, initiating transcription of target genes (Izrailit and Reedijk 2012).

The contribution of aberrant Notch signaling to breast cancer was first noted in the murine mammary gland, where the *Notch4* gene (also known as the *Int-3* locus) was identified as a common proviral integration site in mouse mammary tumor virus (MMTV)–induced mammary tumors (Gallahan and Callahan 1987; Gallahan et al. 1987). Insertion of the provirus into the *Notch4* locus was found to lead to overexpression of a truncated form of NOTCH4 which was structurally similar to the activated NOTCH4 intracellular domain ($N4^{IC}$). Transgenic mice expressing $N1^{IC}$, $N3^{IC}$, or $N4^{IC}$ driven by either the MMTV or

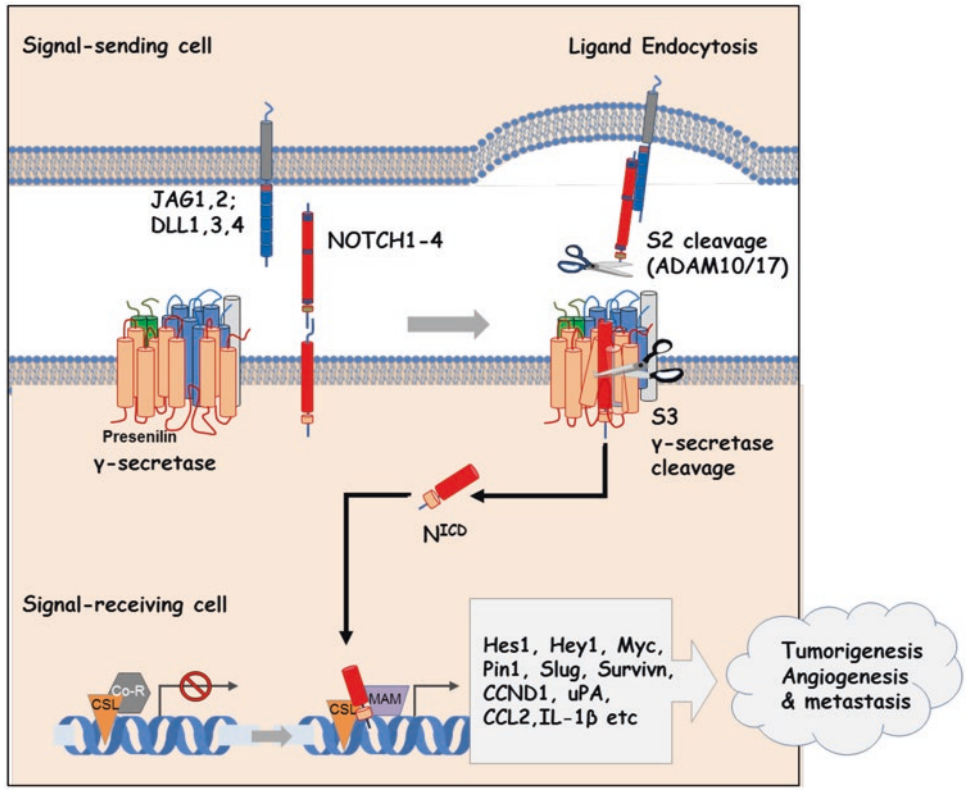

Fig. 12.2 The canonical Notch signaling pathway

whey acidic protein (WAP) promoters recapitulate the oncogenic potential of MMTV infection (Gallahan and Callahan 1987; Gallahan et al. 1987). These transgenic animals have similar phenotypes, exhibiting impaired ductal and lobuloalveolar mammary gland development, followed later by the emergence of mammary gland tumors (Hu et al. 2006; Kiaris et al. 2004).

The first clue that Notch may be aberrantly activated in primary human breast cancer came from a study demonstrating increased expression of NOTCH1 in four breast tumors that overexpressed H-RAS (Weijzen et al. 2002). The authors demonstrated that downregulation of NOTCH1 led to reduced proliferation of RAS-transformed human cells, and that abrogation of RAS signaling prevented upregulation of $N1^{IC}$. These findings suggested that $N1^{IC}$ functions as a downstream mediator of oncogenic RAS and is necessary to maintain the neoplastic phenotype of RAS-transformed cells.

Mechanistically, unlike T-cell acute lymphoblastic leukemia where more than 50% of cases harbor gain-of-function mutations in the extracellular heterodimerization and/or the C-terminal PEST domains of NOTCH1 (Weng et al. 2004), somatic mutations in *Notch* genes are rare in solid cancers, including breast cancer (Lee et al. 2007). In breast cancer, Notch is primarily activated through upregulation of Notch ligands and/or receptors, predominantly through transcriptional (Weijzen et al. 2002) and posttranslational mechanisms (Foltz et al. 2002; Fryer et al. 2004; Jehn et al. 2002; McGill and McGlade 2003; Mukherjee et al. 2005; Oberg et al. 2001; Rustighi et al. 2009). For example, ligand and/or receptor transcriptional upregulation can occur in response to hypoxia, IL-6, RAS, or WNT activation (Weijzen et al. 2002). Posttranslational mechanisms include prolyl-isomerase PIN1-mediated enhancement of γ-secretase activity and enhanced NOTCH1 activation (Rustighi et al. 2009).

Importantly, Notch activation in ductal carcinoma in situ (DCIS) predicts early recurrence (Farnie et al. 2007) and in invasive breast cancer is associated with progression. Moreover, there is a synergistic effect of high-level JAG1 and NOTCH1 co-expression on overall survival in breast cancer (Reedijk et al. 2005, 2008; Dickson et al. 2007; Parr et al. 2004). In the next sections, we review how Notch influences many of the "hallmarks of cancer" to promote breast cancer.

Notch and Breast Cancer Subtype

Elevated expression of Notch ligands and receptors is associated with poor prognosis pathologic features, and poor outcome in breast cancer patients (Reedijk et al. 2005, 2008; Parr et al. 2004; Lee et al. 2008a; Dickson et al. 2007). The association between Notch activation and upregulation of the apoptosis inhibitor and cell cycle regulator SURVIVIN is exclusive to $ER^-/HER2^-$ basal-like breast cancer cells (Lee et al. 2008a, b). In a recent report, the importance of Notch-mediated signaling in the proliferation of $HER2^-$, and not $HER2^+$ breast cancer cells was shown (Yamaguchi et al. 2008). In this study four of five $HER2^-$ breast cancer cell lines for which this association was established were also ER^-/PR^- (i.e., TNBC). BRCA1-mutant breast cancers, which are predominantly ER^- and of the basal-like subtype, are associated with elevated JAG1 expression compared to their BRCA2 (predominantly luminal) counterparts (Bane et al. 2008). Consistent with these findings, cell line data confirm an association between elevated JAG1 and the TNBC/BLBC subtype (Cohen et al. 2010).

Further studies indicate that Notch may be activated in breast cancer cells where ER or HER2 signaling are medically silenced. While estradiol treatment of ER^+ cells inhibits $N1^{IC}$ nuclear levels and Notch activity (these effects are in part mediated by inhibition of γ-secretase (Rizzo et al. 2008)), treatment of these cells with selective estrogen receptor modulators (SERMs) reverses this effect. Similarly, HER2 overexpression suppresses NOTCH1 activity in $HER2^+$ breast cancer cell lines, while treatment with trastuzumab reactivates Notch signaling (Osipo et al. 2008).

Taken together, these findings suggest that in the absence of the growth stimulatory effects of estrogen or HER2 (i.e., in TNBC/BLBC or SERM-/trastuzumab-treated breast cancers), Notch activation may provide a compensatory, growth-promoting signal. These discoveries make the Notch signaling pathway an attractive therapeutic target in TNBC/BLBC or drug-resistant ER^+ or $Her2^+$ breast cancers.

Notch and Tumor-Initiating Cells

Similar to the way in which Notch may regulate self-renewal of normal mammary somatic stem cells (SCs), Notch is a regulator of tumor-initiating cells (TICs), cells endowed with self-renewal capacity, and the ability to repopulate a tumor (Dontu et al. 2003, 2004). In breast cancer, TICs were originally identified as lineage-negative (lin^-) $CD44^+/CD24^{-/low}$ cells (Al-Hajj et al. 2003). These cells can form tumors in non-obese, diabetic/severe combined immunodeficient (NOD/SCID) mice when as few as 200 cells are transplanted into the cleared mammary fat pad. In comparison, not even 10,000 unselected cells are capable of the same. Additional work has identified the aldehyde dehydrogenase (ALDH)–positive subgroup of $CD44^+/CD24^{-/low}/lin^-$ cells as further enriched for TICs (Ginestier et al. 2007). Based on methodology to propagate SC within mammospheres, Ponti et al. have described in vitro culture of putative breast TICs as multicellular tumorspheres (Ponti et al. 2005). Analogous to the mammosphere–SC relationship, tumorspheres are surrogates for TICs. Tumorspheres contain undifferentiated cells that are capable of self-renewal and the generation of daughter spheres, and cells that can differentiate along pathways to generate ductal and myoepithelial mammary lineages. Implicating Notch in TIC expansion, studies in DCIS-derived tumorspheres showed that

the efficiency of tumorsphere production was reduced through Notch inhibition by either GSI or an anti-NOTCH4 monoclonal antibody (Farnie et al. 2007). Using tumorsphere culture of primary breast cancers and breast cancer cell lines, NOTCH3 and JAG1 were identified as a key regulators of TIC renewal and hypoxia survival (Sansone et al. 2007a, b), providing rationale for the development of Notch-based therapeutics that target TICs.

Notch and Breast Cancer Cell Proliferation and Survival

In human breast cancer, the *CCND1* gene is a direct transcriptional target of NOTCH1 and NOTCH3 (Cohen et al. 2010). CYCLIN D1 is a key cell cycle regulator necessary for advancement through the G1 phase of the cell cycle; it plays an important pathogenetic role in breast cancer (Arnold and Papanikolaou 2005; Sherr and Roberts 1999). Accordingly, JAG1 downregulation reduces CYCLIN D1 expression and inhibits cell cycle progression through the G1/S checkpoint. Furthermore, CYCLIN D1 and JAG1 expression correlate in TN breast cancer expression datasets, suggesting a model whereby JAG1 promotes CYCLIN D1–mediated proliferation of TN breast cancers. Additional cell cycle regulatory proteins including CYCLIN A and B1 have been implicated as targets in Notch-mediated cell cycle progression in breast cancer (Rizzo et al. 2008). Further, an antiapoptotic role of Notch was demonstrated in breast cancer through AKT (Meurette et al. 2009), which provides resistance to numerous apoptotic stimuli. SURVIVIN, a member of the inhibitor of apoptosis protein family, is overexpressed in a wide spectrum of malignancies, including breast cancer (Altieri 2008), and is a direct transcriptional target of Notch (Lee et al. 2008b). A meta-analysis of multiple human breast cancer microarray datasets has revealed a statistically significant relationship between high NOTCH1 expression levels, increased SURVIVIN expression, and poor overall survival (Lee et al. 2008a), further supporting the antiproliferative and proapoptotic potential of Notch-based therapeutics.

Notch and Epithelial–Mesenchymal Transition

Epithelial–mesenchymal transition (EMT) is directly associated with malignant progression and dissemination in breast cancer (Vincent-Salomon and Thiery 2003). Overexpression of $N1^{IC}$ induces the expression of SNAIL, a repressor of E-CADHERIN and an inducer of EMT (Timmerman et al. 2004), suggesting that Notch promotes metastases. SLUG is similarly a direct Notch target (Leong et al. 2007), and like SNAIL can represses E-CADHERIN and induce EMT in breast cancer (Martin et al. 2005). Fitting with these findings, Leong et al. have shown that JAG1 and SLUG expression correlate in primary breast cancer, and that blocking Notch in breast cancer xenografts restores E-CADHERIN expression, inactivates β-CATENIN, and blocks growth and metastases in a SLUG-dependent fashion (Leong et al. 2007). These findings suggest that ligand-induced Notch activation promotes EMT and metastases in breast cancer.

Notch and the Breast Cancer Microenvironment

The Breast Cancer Microenvironment at a Glance

A tumor is a disorganized organ where proliferating malignant cells recruit and potentially corrupt "non-malignant" cells. Intracellular interactions are supported by a complex network of cytokines, growth factors, and matrix remodeling enzymes. Breast cancers likely arise from the same epithelial components that give rise to the mammary ducts, which consist of a luminal epithelial cell layer underlined by myoepithelial cells. These structures are surrounded by a basement membrane matrix and supported by a stroma consist-

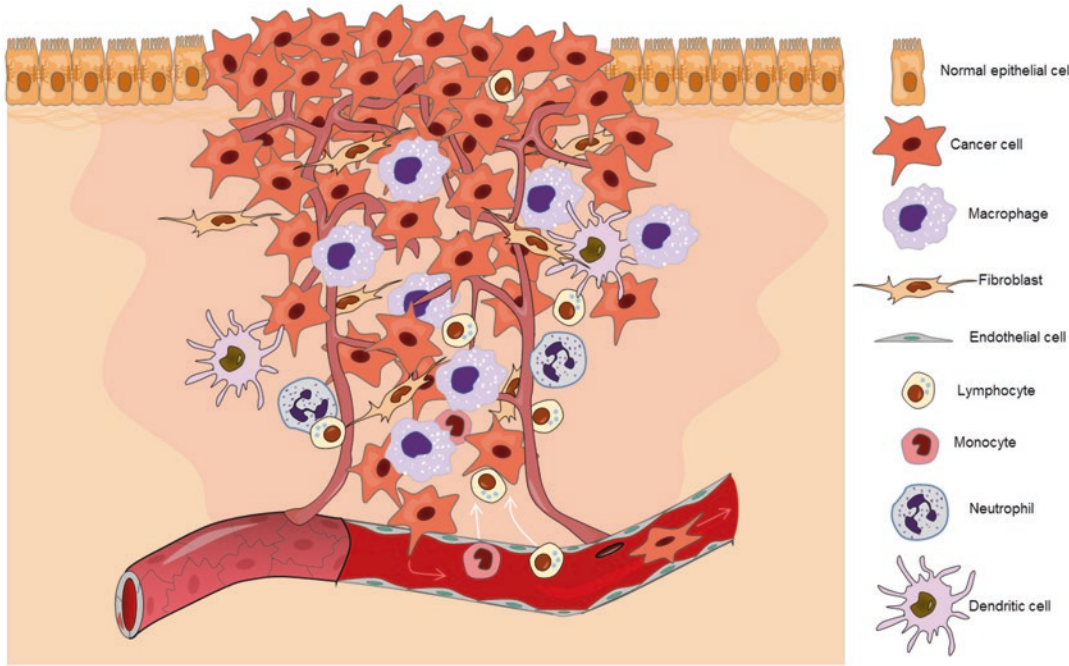

Fig. 12.3 The breast cancer tumor microenvironment

ing of varying cell types. In a breast cancer, the malignant compartment is similarly supported by nonmalignant cells of diverse lineages, such as angiogenic vascular endothelial cells, cells of both the innate and adaptive immune system, and fibroblasts (Fig. 12.3) (Grivennikov et al. 2010; Hanahan and Coussens 2012; Hanahan and Weinberg 2011; Mantovani et al. 2008). These nonmalignant components influence malignant progression, and in some cases are complicit in therapeutic resistance. Therefore, identifying the mechanisms that regulate the patterns of nonmalignant tumor infiltrates and their bidirectional crosstalk with tumor cells is paramount. Notch signaling is active in these cellular compartments and regulates cellular communication within the TME. This is mediated through not only homo- or heterotypic juxtacrine signaling but also paracrine signaling through cytokines, matrix remodeling enzymes, and growth factors (Shen et al. 2017; Reedijk 2012; Shimizu et al. 2011; Wellenstein and de Visser 2018). This chapter will introduce recent findings that implicate Notch in crosstalk between various TME compartments and explores translating this understanding into the development of novel therapies.

Notch and Tumor Angiogenesis

Rapid tumor growth demands an abundant supply of nutrients and oxygen together with the swift removal of metabolic waste from TME. This elevated level of blood–tissue exchange is sustained by a dense microvasculature developed through pathological tumor angiogenesis. Compared to the microvasculature found in normal tissues, tumor microvessels have thin walls with defective endothelium, partially due to underdeveloped endothelial cell–cell junctions and discontinuous pericytes and smooth muscle (Hashizume et al. 2000; Morikawa et al. 2002). As a consequence, the endothelium is hyperpermeable. This plays an essential role in tumor progression by facilitating cell transmigration (inflammatory cell intravasation into tumor and cancer cell extravasation/metastasis) and plasma accumulation in the TME.

The angiogenic process is characterized by highly orchestrated cell–cell communication requiring multiple juxtacrine pathways, growth factors, adhesion molecules, and matrix proteins (Mollen et al. 2018; Piulats and Mitjans 2008). Among these, vascular endothelial growth factor (VEGF) and its receptor are indispensable mediators of angiogenesis in cancer (Carmeliet 2005). Accordingly, VEGF is highly expressed in breast cancer (Anan et al. 1996) and its expression level correlates with tumor grade (Dvorak et al. 1995). Notch is another essential mediator of angiogenesis and vasculogenesis (Roca and Adams 2007; Gridley 2007; Siekmann and Lawson 2007). For example, Notch specifies the leading "tip" endothelial cells and the growing "stalk" cells to form new capillary networks (Gridley 2007; Siekmann and Lawson 2007). The stability of microvessels is also regulated by Notch through vascular mural cell function.

During angiogenesis there exists an intricate interplay between the Notch and VEGF systems that controls the source of the ligands and receptors of these pathways. While the Notch pathway regulates the expression of VEGF receptors VEGFR-1 and VEGFR-2 (Suchting et al. 2007; Taylor et al. 2002; Harrington et al. 2008; Funahashi et al. 2010; Outtz et al. 2010; Shawber et al. 2007), reciprocally, VEGF coordinates tumor endothelial expression of DLL4, which serves as a negative-feedback regulator of vascular growth (Noguera-Troise et al. 2006). VEGF, while traditionally attributed to tumor cells (Hoeben et al. 2004), can also be supplied by TAMs, which are endowed with the capacity to produce a multitude of proangiogenic factors (Classen et al. 2009; Noonan et al. 2008; Leibovich et al. 1987; Huang et al. 2004). In fact, the proangiogenic activity of macrophages depends on their Notch-activated state and is essential for them to promote the formation of anastomotic bridges between DLL4-positive endothelial tip cells (Outtz et al. 2011). In turn, contact between macrophages and endothelial cells allows for Notch-mediated induction of sprouting angiogenesis (Tattersall et al. 2016). Tumor cells can also promote angiogenesis in a fashion that depends on the Notch pathway. For example, in head and neck squamous cell carcinoma, JAG1 expression in tumor cells activates Notch and vascular network formation in neighboring endothelial cells (Zeng et al. 2005). In another example, breast cancer cells are an important source of Notch ligand, activating Notch3 which is highly expressed in blood vessels, and implicated in tumor angiogenesis (Callahan and Egan 2004).

While most efforts to block tumor angiogenesis have focused on blocking the VEGF pathway, tumors exhibit widely varying susceptibility to VEGF blockade, with some tumors being completely insensitive (Lupo et al. 2016). In some VEGF blockade-insensitive tumors, because of its negative feedback role, blockade of DLL4 results in an increase in nonproductive tumor angiogenesis and in paradoxical decreased tumor growth. Clearly, Notch blockade holds promise as a suppressor of cancer angiogenesis and tumor growth.

Notch and the Extracellular Matrix

The extracellular matrix (ECM), commonly defined as the noncellular components of the TME, provides both a structural scaffold for cellular components and a biochemical reservoir for growth factors and chemokines that are necessary for tumor progression. ECM remodeling is spatiotemporally regulated by ECM proteinases, matrix metalloproteinases (MMPs), and plasminogen activator (PA)–plasmin systems (Jablonska-Trypuc et al. 2016; Lu et al. 2011), which not only facilitate tumor spreading, but also activate and release growth factors that contribute to tumor proliferation, angiogenesis, and metastasis (Discher et al. 2009; Deryugina and Quigley 2006).

Notch contributes to ECM remodeling by regulating the expression of proteinases. MMPs, which are secreted and activated primarily by malignant cells, TAMs, and cancer-associated macrophages (CAFs), are the main ECM enzymes responsible for ECM protein degrada-

tion (Poltavets et al. 2018). In BLBC, NOTCH1 activates NF-κB signaling and upregulates the expression of the NF-κB target genes, MMP-2 and MMP-9 (Li et al. 2014). Expression data in both breast cancer cell lines and primary tumors demonstrate an association between elevated expression of JAG1, urokinase PA (uPA), and the basal-like breast cancer subtype. In fact, a CBF-1 binding site has been identified in the uPA promoter that is required for direct transcriptional regulation by Notch (Shimizu et al. 2011). uPA binds to its receptor (uPAR), which facilitates conversion of plasminogen to plasmin. Plasmin either directly, or indirectly through metalloproteinases (MMP), degrades components of the extracellular matrix, contributing to cancer cell invasion and metastases. Members of the plasminogen activator system including uPA have been validated as markers of recurrence, high metastasis risk, and death in breast malignancy (Annecke et al. 2008). These data suggest that JAG1-induced Notch activation results in breast cancer progression through upregulation of the plasminogen activator system, and these findings directly link these two poor prognostic pathways. In a follow-up study, the Notch-regulated uPA–plasmin axis was found to be responsible for cleavage and activation of latent transforming growth factor-beta (TGF-β) derived from TME components, such as TAMs (Liu et al. 2018; Yang and Zhang 2017) and CAFs (Ren et al. 2018). Once activated, TGF-β stimulates the TGF-β receptor 1 (TGFβR1), another Notch target gene in breast cancer cells, which activates JAG1–Notch signaling, closing a feedback loop resulting in tumor cell invasive motility (Shen et al. 2017).

Notch and Cellular Stress

The hypermetabolic demands of rapidly proliferating tumor cells can be accompanied by oxidative and nutrient stress. Stress further serves as a pathological selective pressure to promote tumor aggressiveness (Ackerman and Simon 2014). A better understanding of the molecular pathways that allow tumor cells to thrive in the midst of nutrient and oxygen deprivation is an obvious route to generate more effective therapies.

In breast cancer, hypoxia potentiates Notch signaling. This results in decreased E-cadherin expression, accompanied by increased cell migration and invasion (Chen et al. 2010). The underlying mechanism may involve the accumulation of hypoxia inducible factor-1 alpha (HIF-1α) and hypoxia inducible factor-2 alpha (HIF-2α), which synergize with the Notch coactivator mastermind-like transcriptional coactivator 1 (MAML1) to upregulate SLUG and SNAIL and to downregulate E-cadherin (Chen et al. 2010). Hypoxia can also upregulate p66SHC or IL-6, two proteins that can induce NOTCH3 and ERK-dependent JAG1 expression in breast cancer cells (Sansone et al. 2007a, b).

Hypoxia-induced Notch activation is not limited to the aforementioned mechanisms. Through large-scale chemical kinase inhibitor and kinase-specific siRNA-based screens the pseudokinase tribbles homolog 3 (TRB3) was identified as a critical regulator of JAG1-induced Notch activation and tumor growth through its control of MAPK and TGF-β signaling pathways (Izrailit et al. 2013). TRB3 is a stress sensor in physiologic conditions such as fasting, where it promotes hepatic glucose output by binding to and interfering with insulin-induced AKT phosphorylation (Du and Ding 2009). Likewise, TRB3 can act as a sensor of stress in pathologic conditions, including cancer. TRB3 is upregulated in cancer in response to hypoxia or starvation, promotes tumor cell survival, and predictably is linked to poor outcome (Wennemers et al. 2011; Miyoshi et al. 2009; Bowers et al. 2003; Ord et al. 2007; Schwarzer et al. 2006). As a scaffolding protein, TRB3 interacts with numerous molecules, such as E3 ligases, receptor kinases, transcription factors, and the substrates that regulate their activation, subcellular localization, or degradation (Izrailit et al. 2013; Hua et al. 2011; Ohoka et al. 2005; Qi et al. 2006). In a follow-up study to further clarify the mechanism by which TRB3 mediates the effect of hypoxia on Notch, the deubiquitinating enzyme USP9x (ubiquitin-specific peptidase 9 X-linked) was identified as a novel

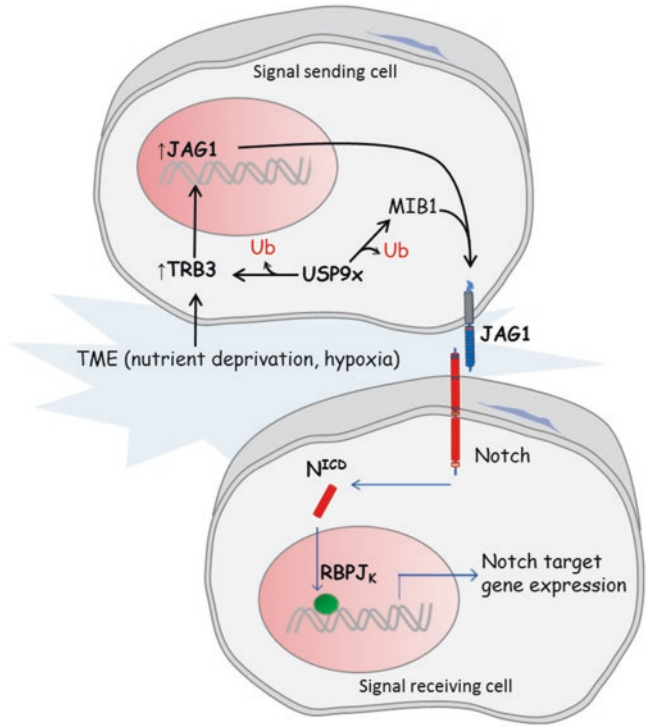

Fig. 12.4 Stress in the TME upregulates TRB3, which together with USP9x activates JAG1 in signal-sending breast cancer cells

interacting partner in a stress-induced multiprotein complex with TRB3 and the E3 ligase, Mind Bomb (MIB). MIB is required to activate JAG1 (Itoh et al. 2003). Here, USP9x regulates JAG1 expression and activation through deubiquitination and stabilization of TRB3 and MIB1 respectively, in the signal-sending cell during paracrine Notch activation (Fig. 12.4) (Izrailit et al. 2017). These findings identify TRB3 and USP9x as potential therapeutic targets. Supporting the critical role of USP9x as a positive regulator of the Notch pathway, USP9x is associated with BLBC cell migration and metastases (Dupont et al. 2009; Xie et al. 2013). Also, mutations in the *Usp9x* gene and USP9x protein overexpression have been implicated in other cancers such as B-cell malignancies (Zhou et al. 2015), colorectal carcinoma (Harris et al. 2012), and lung cancer (Wang et al. 2015).

The case for Notch as a survival pathway induced by hypoxia and starvation in cancer is clear. The related pathways that mediate this effect provide further potential avenues to therapeutically target Notch in breast cancer.

Notch Signaling and the Immunoenvironment

Overview of the Breast Cancer Immunoenvironment

Breast cancer is characterized by a highly inflammatory microenvironment consisting of immune cells of both the innate and adaptive systems. Although not an all-inclusive list, the immune infiltrate consists of TAMs, neutrophils, myeloid derived suppressor cells (MDSC), dendritic cells (DC), natural killer cells (NK), regulatory T-cells (Tregs), CD4+ T-cells, and cytotoxic CD8+ T-cells (CTL) (Polyak 2011; Esquivel-Velazquez et al. 2015). The pattern of immune infiltration, which differs between breast cancer subtypes, is directly related to clinical outcome and treatment response. In ER-negative breast cancer, the majority of immune cells are TAMs and Tregs, while cytotoxic CD8+ and CD4+ helper T-cells are found in smaller proportions, defining a so-called "cold" or immunosuppressed TME. By promoting angiogenesis, tumor cell migration

and invasion, and suppression of antitumor immunity (Chen et al. 2005; Qian and Pollard 2010; Mahmoud et al. 2012), TAMs have emerged as an important factor associated with poor prognosis in BLBC (Ali et al. 2016). By similarly suppressing antitumor immunity, Treg infiltration is associated with relapse and an unfavorable prognosis in both ER-negative and ER-positive breast cancer (Bates et al. 2006; Wang et al. 2017). Predictably, those tumors with the highest proportion of CD8+ and activated memory T-cells have the most favorable outcome (Ali et al. 2016; Bates et al. 2006; Denkert et al. 2010; Watanabe et al. 2010; Mahmoud et al. 2011; Medrek et al. 2012; Ali et al. 2014; Svensson et al. 2015; Dieci et al. 2018). In ER-positive breast cancers, neutrophils and NK cells comprise a strong proportion of immune infiltrates (Ali et al. 2016). Therefore, identifying the pathways that regulate immune infiltration, in particular TAMs, will illuminate novel immunotherapeutic avenues to treat breast cancer.

Notch Signaling and TAMs

TAMs are heterogeneous, displaying functional differences as well as differences in the cells from which they originate. Their heterogeneity is driven by several factors including the tumor type in which they reside, tumor stage, and their location within the tumor. TAMs can be derived from monocytes recruited from the circulation or they can originate from tissue-resident macrophages. Compared to brain tumors for example, where TAMs originate from both recruited monocytes and resident microglia cells, in breast cancer TAMs are predominantly derived from newly recruited monocytes (Franklin et al. 2014; De Palma 2016).

Macrophages that promote inflammation are classically activated, M1-polarized macrophages. This distinguishes them from alternatively activated, anti-inflammatory, M2-polarized macrophages, which secrete specific cytokines (IL-6, IL-10) and growth factors (TGF-β, VEGF, FGF2). M2 macrophages represent the majority of TAMs and encourage an anti-inflammatory and immunosuppressive TME. However, classifying TAMs and their activities into one of these subsets may be somewhat of an oversimplification. Emerging in vitro and in vivo studies have shown the presence of diverse hybrid phenotypes of macrophages overlapping with each other in gene expression and function (Mosser and Edwards 2008; Roszer 2015; Xue et al. 2014; Gautier et al. 2007). Using an in vivo model of 4T1 mammary carcinoma, TAMs were found with both pro-inflammatory and anti-inflammatory phenotypes, suggesting highly complicated and context-dependent mechanisms that regulate their phenotype and activation within the TME (Movahedi et al. 2010).

Notch receptors and ligands are detected in macrophages including TAMs (Franklin et al. 2014; Wang et al. 2010). In response to pro-inflammatory cues, such as lipopolysaccharide (LPS), with or without interferon-γ (IFNγ), Notch favors differentiation towards a pro-inflammatory phenotype (Wang et al. 2010; Palaga et al. 2008; Hu et al. 2008; Fung et al. 2007). This is supported by findings in an RBPJκ-deficient mouse model, where canonical Notch signaling promotes an M1-like cell fate by driving expression of interferon regulatory factor 8 (Xu et al. 2012). Additionally, Notch can reprogram mitochondrial metabolism toward oxidative phosphorylation, further endorsing a pro-inflammatory M1 phenotype (Xu et al. 2015). On the other hand, in response to IL-4 or various other pro-inflammatory inducers (Foldi et al. 2016; Zhang et al. 2006; Edwards et al. 2006), Notch activation can induce an anti-inflammatory macrophage phenotype with the expression of M2 genes such as ARG1 (Arginase-1) and IL-10 (Zheng et al. 2016). Notch signaling within tumor cells can also promote the M2 phenotype. In breast cancer, tumoral expression of JAG1 modulates TAM differentiation, favoring the production of IL-10-secreting, anti-inflammatory TAMs (Liu et al. 2017). Similarly, in head and neck squamous cell carcinoma NOTCH1 expression is associated with CD68+ CD163+ M2-like TAMs where γ-secretase inhibition enhances antitumor immunity (Mao et al. 2018). While regulating seemingly contradictory development of both M1 and M2 macrophages,

what has emerged is a model where Notch-driven macrophage differentiation is complex and highly context-dependent. Since TAMs are an essential determinant in tumor development, a deeper understanding of how Notch regulates TAM activity will be a key to developing effective immunotherapies for breast cancer.

Recently it was discovered that Notch activation in tumor cells is instrumental in shaping the tumor innate immunophenotype in BLBC (Shen et al. 2017) (Fig. 12.5). Novel in concept, it was shown that the recruitment of TAMs to the TME is driven by tumor cell Notch-regulated expression of pro-inflammatory cytokines, IL1β and C-C motif chemokine ligand 2 (CCL2) (Shen et al. 2017). As mediators of innate immune response, IL1β and CCL2 are two pleiotropic cytokines that promote tumor growth and metastasis through mechanisms such as myeloid cell recruitment, senescence bypass, angiogenesis, and enhanced invasiveness. In the aforementioned study the *IL1B* gene was identified as a direct transcriptional target of activated Notch (Shen et al. 2017). The resulting proprotein (pro-IL1β) is further activated by caspase-1 which is found in the inflammasome multiprotein complex required to process IL1β (Franchi et al. 2009). While its expression is Notch-independent, caspase-1 is also a hallmark of BLBC, making this breast cancer subtype a "perfect storm" for IL1β production (Shen et al. 2017).

The essential role of CCL2 signaling through C–C chemokine receptor type 2 (CCR2) in TAM recruitment to breast cancer is supported by the observation of decreased TAM infiltration into tumors in a mouse mammary tumor model (MMTV-PyMT) in which the *CCR2* gene has been deleted (Franklin et al. 2014). Additionally, deletion of RBPJκ in macrophages results in loss of CCR2 and other TAM markers, signifying another mechanism by which Notch regulates TAM chemotaxis in the TME (Franklin et al. 2014). In addition to its role as a recruiter of TAMs, CCL2 stimulation also shapes macrophage polarization. CCL2 enhances anti-inflammatory cytokine IL-10 production in macrophages pushing them toward an M2-like phenotype (Sierra-Filardi et al. 2014). Conversely,

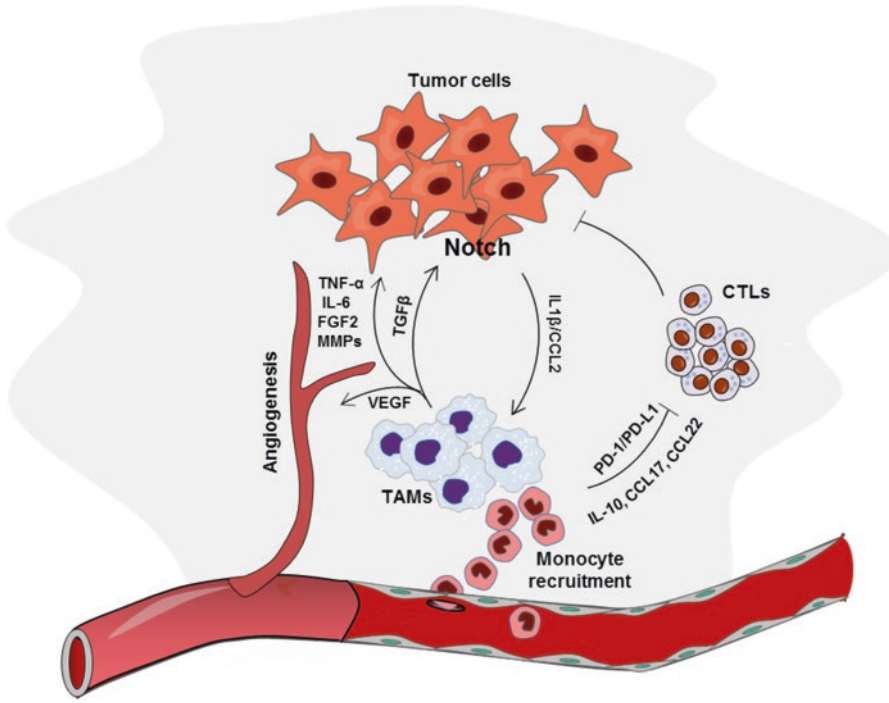

Fig. 12.5 Notch signaling shapes the immunophenotype of breast cancer

CCL2 blockade leads to increased expression of genes and cytokines associated with M1 polarization (Sierra-Filardi et al. 2014). Consistent with this, CCR2-deficient macrophages show an M1-polarized gene expression profile (Sierra-Filardi et al. 2014).

As a potent immunosuppressive factor in both innate and adaptive immunity, TGF-β is produced by several components of the TME including TAMs, CAFs, and osteoclasts. Coming full circle, TGF-β promotes JAG1 expression and Notch signaling in tumor cells (Shen et al. 2017; Sethi et al. 2011). By regulating the expression of TGFβR1 on tumor cells (Shen et al. 2017; Zhou et al. 2016), Notch sensitizes tumor cells to activation by TAMs, closing a Notch-dependent paracrine signaling loop between these two cell types (Shen et al. 2017). By using a novel transgenic mouse model where Notch activity can be precisely regulated in mammary epithelial cells and in spontaneously occurring breast carcinoma, a causative role for Notch in cytokine expression and macrophage infiltration in the BLBC TME has been confirmed (Shen et al. 2017).

Thus far, several elegant studies suggest that targeting either the IL-1β/inflammasome or the CCL2-CCR2 signaling axes may provide novel approaches to treat breast cancer.

Notch Signaling and CD8$^+$ T-Cells

As a regulator of binary cell fate, Notch is essential to T-cell differentiation from CD34+ hematopoietic progenitor cells, at the cost of B-cell lineage differentiation (Kyoizumi et al. 2017; Benne et al. 2009; Radtke et al. 2013). TME infiltration by CD8+ T-cells is a strong prognostic factor for a positive outcome in solid tumors, including breast cancer (Ali et al. 2016; Gajewski et al. 2013). In that context, Notch has a further influence over CD8+ T-cell destiny through DLL1-mediated NOTCH1 or NOTCH2 signaling, resulting in the expression of IFNγ and granzyme B, markers of activated cells (Cho et al. 2009). Supporting this, deletion of *Notch2* in CD8+ T-cells results in tumors of increased size and reduces survival in a mouse mammary tumor model (Sugimoto et al. 2010). Conversely, selective stimulation of the DLL1–Notch pathway in T-cells restores T-cell activity and inhibits tumor growth (Huang et al. 2011). Although these findings indicate that Notch activates CD8+ T-cells toward tumoricidal activity, Notch can also suppress tumor-infiltrating CD8+ T-cell activity by upregulation of PD-1 (programmed cell death 1) (Yu et al. 2018; Mathieu et al. 2013). In fact, Notch1 inhibition can enhance immunotherapy efficacy in melanoma by reducing immune suppressive MDSCs and Tregs in the TME, while increasing functional CD8+ T-cell infiltration (Qiu et al. 2018). These somewhat contradictory findings suggest a context-dependent effect of Notch on CD8+ T-cell function in TME.

Notch and Cancer-Associated Fibroblasts

As the predominant cell type responsible for "reactive stroma" (Kalluri and Zeisberg 2006; Pietras and Ostman 2010; Orimo et al. 2005), cancer-associated fibroblasts (CAFs) are vital to breast cancer development and metastasis (Dvorak et al. 2018; Houthuijzen and Jonkers 2018). Similar to myofibroblasts in wound healing, CAFs acquire an activated phenotype (Shimoda et al. 2010; Sappino et al. 1988) characterized by elevated expression of α-smooth muscle actin, ECM molecules, tenascin-C, MMPs, growth factors, and cytokines (Kalluri and Zeisberg 2006; Rodemann and Muller 1991; Bhowmick et al. 2004; Serini and Gabbiani 1999; De Wever et al. 2004). CAFs extracted from human breast carcinomas promote MCF-7-RAS breast cancer cell growth with greater efficiency than do their normal mammary fibroblast counterparts (Orimo et al. 2005). This is mediated by CAF-derived stromal cell-derived factor 1 which not only induces angiogenesis by recruiting endothelial progenitor cells, but also enhances tumor growth directly through interaction with its cognate C–X–C chemokine receptor type 4 found on cancer cells (Orimo et al. 2005). CAFs isolated from metastatic breast cancer also express IL6 which promotes tumor growth and invasiveness through paracrine induction of Notch in cancer cells (Studebaker et al. 2008).

CAFs contribute to the maintenance of TICs via a number of mechanisms (Giannoni et al. 2010). For example hepatocyte growth factor (HGF) secreted by CAFs binds its receptor, c-MET on colon carcinoma cells to activate the WNT signaling pathway and the TIC phenotype (Vermeulen et al. 2010). Notch has also been implicated in CAF-induced cancer cell "stemness." In breast cancer, CAF-secreted CCL2 confers the stem cell phenotype on breast cancer cells by activating Notch (Tsuyada et al. 2012). Further, therapy resistance pathways are potentially regulated by JAG1/Notch3-mediated crosstalk between CAFs and breast cancers through the expansion of $CD44^{+}CD24^{low}$ tumor-initiating and therapy-resistant cells (Boelens et al. 2014).

Here again, CAFs, a key tumor-promoting component of the TME, depend on Notch to execute their pathologic activities.

Conclusions and Therapeutic Perspectives

Since the discovery by Gallahan and Callahan (1987) more than two decades ago, significant progress has been made toward understanding the pathologic role of Notch in the development and progression of breast cancer and other malignancies. Consistent with its pleotropic effects in normal development and tissue maintenance, aberrant Notch supports several hallmarks of human cancer progression, including TIC maintenance, cell fate specification and proliferation, inhibition of apoptosis, and promotion of invasion and metastases. Perhaps more profound is the recent recognition of the essential role of Notch in a vast panorama of juxtacrine and paracrine communications within the TME that support angiogenesis, activated stromal cells, adaptability to cellular stress and critically, the shape of the innate and adaptive immune landscape. With that in mind, various therapeutic strategies have been developed to target Notch at each activation step within the pathway, such as Notch receptor (tarextumab, Notch1 decoy constructs) and ligand (DLL4, CTX014) antagonists, γ-secretase inhibitors (DAPT, MRK-003, MK-0752, RO4929097), and compounds targeting the transcription complex (Reedijk 2012; Ntziachristos et al. 2014; Takebe et al. 2015), with compelling preclinical success.

Despite these successes, Notch inhibitors have been slow to come to clinic, mainly because they can induce significant side effects (Yuan et al. 2015). Considering both that the aforementioned anti-Notch approaches do not specifically target Notch in cancer cells, and that in some contexts Notch may have antitumoral effects, extra layers of complexity must be considered to mitigate and minimize potential collateral damage. With that in mind, immunotherapies targeting immune checkpoint proteins such as programmed death 1 (PD-1) receptor and ligand (PD-L1/2), represent a major breakthrough to reboot suppressed TILs. We hypothesize that by recruiting TAMs, Notch indirectly suppresses TILs. Indeed, despite TNBC being 60% positive for PD-L1, immune checkpoint blockade (ICB) has only shown a 20% response rate in this subtype – a subtype where 80% of cases are highly infiltrated by TAMs (Vonderheide et al. 2017). These observations suggest that anti-Notch strategies such as anti-TAM immunotherapy (IL1β or CCL2 antagonists) may overcome ICB resistance, to treat BLBC.

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Notch Pathway: A Journey from Notching Phenotypes to Cancer Immunotherapy

13

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Abstract

Notch is a key evolutionary conserved pathway, which has fascinated and engaged the work of investigators in an uncountable number of biological fields, from development of metazoans to immunotherapy for cancer. The study of Notch has greatly contributed to the understanding of cancer biology and a substantial effort has been spent in designing Notch-targeting therapies. Due to its broad involvement in cancer, targeting Notch would allow to virtually modulate any aspect of the disease. However, this means that Notch-based therapies must be highly specific to avoid off-target effects. This review will present the newest mechanistic and therapeutic advances in the Notch field and discuss the promises and challenges of this constantly evolving field.

Keywords

Notch · Drosophila · Cancer · Notch-targeting therapies · Endocytosis · Glycosylation · Metabolism · Immunotherapy · Synthetic biology

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The Basics of Notch Pathway

Notch discovery tracks back to more than 100 years ago when the geneticist John S. Dexter first observed "notches" in the veins of *Drosophila melanogaster* wings and Thomas Hunt Morgan identified the fly Notch mutant alleles (Dexter 1914; Morgan 1917). Spyros Artavanis-Tsakonas and Michael Young were the firsts to identify *Notch* gene, and link the notching fly wing phenotype to alterations in the *Notch* locus (Kidd et al. 1986; Wharton et al. 1985). As soon as a decade after its discovery, Notch made its entry in different fields from developmental to cancer biology. As we will discuss in this chapter, nowadays Notch is the protagonist of some of the most cutting-edge fields, including immunotherapy and synthetic biology.

Notch is a developmental signaling pathway evolutionary conserved across metazoans (Animalia kingdom) from *Drosophila* to humans. While only one Notch receptor and two ligands (Delta and Serrate) are present in flies, evolution provided humans with four Notch receptors (Notch1–4) and five canonical ligands (Delta-like ligand 1, 3, 4 [DLL1, DLL3, DLL4], Serrate-like ligand Jagged 1–2 [JAG1 and JAG2]) (Fleming 1998; Gordon et al. 2008; Aster et al. 2017). Notch receptors are single-pass transmembrane proteins and consist of different conserved functional domains (Fleming 1998; Gordon et al. 2008; Aster et al. 2017). The

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extracellular domain of Notch is composed by epidermal growth factor(EGFs)–like repeats, the number of which varies among species and different Notch receptors. Two functional domains are present in the extracellular region, the ligand-binding domain (EGF 11–12), which mediates the interaction with ligands, and the Abruptex domain (EGF 24–29), the function of which is not yet clear. The extracellular region is followed by the Negative Regulatory Region (NRR), which masks a cleavage site (S2) important for Notch activation, the heterodimerization domain (HD), and the transmembrane spanning region of the receptor. The intracellular domain of Notch (NICD) consists of the RBPJκ-associated molecule region, the Ankyrin domain and transactivation domain, which are involved in the transcriptional activation of Notch target genes. Finally, the C-terminal domain, known as proline, glutamic acid, serine, and threonine-rich (PEST) domain, ensures the stability of NICD. In mammals, Notch receptors are cleaved in S1 site in the HD domain in the Golgi and presented at the cell surface as noncovalently-linked heterodimers consisting of an extracellular and a transmembrane unit. Notch pathway activation starts with the binding of Notch receptor to its transmembrane ligands presented by neighboring cells, a process known as transactivation (Kopan and Ilagan 2009; Bray and Gomez-Lamarca 2018). This exposes the proteolytic cleavage site, S2, in the NRR, which is cleaved by ADAM metalloproteases. A subsequent cleavage, S3, mediated by γ-secretase occurs in the transmembrane region, releasing NICD. NICD translocates to the nucleus where together with the DNA-binding factor RBPJκ (also known as CSL in mammals or Suppressor of Hairless in *Drosophila*) and the coactivators Mastermind-like (MAMLs) triggers the transcription of target genes.

Notch is a master regulator of cell fate and tissue homeostasis and the variety of outcomes of Notch signaling in these processes is astonishing. Notch acts as an oncogene or tumor suppressor, thus either promoting proliferation or apoptosis, in different tissues or subset of cells or cellular contexts (Ntziachristos et al. 2014; Bray 2016). Notch activation has different and sometimes opposite outcomes in developmental processes depending on when and how Notch is activated (Bray 2016; Artavanis-Tsakonas et al. 1999). Given that Notch core pathway seems relatively simple, how can we explain its versatility? The interaction between different Notch receptors and ligands does change the outcome of Notch; however, this is not enough to sustain its multiple and versatile functions. It is becoming clear that Notch pathway relies on a complex regulation, which goes beyond ligand–receptor interactions from the maturation in the Golgi/ER, to the cell membrane, endosomes, and nucleus. One of the easiest explanations of Notch vary outcomes is that in cells with different chromatin states, Notch activates different sets of genes. However possible, it also seems that the kinetic, time, and interaction with enhancers of NICD might be responsible of different transcriptional outcomes (Falo-Sanjuan et al. 2019; Gomez-Lamarca et al. 2018). Further, crosstalks with other pathways within the nucleus and upstream, which have been reported also in disease contexts, might lead to a different outcome (Collu et al. 2014; Gutierrez and Look 2007). Finally, increasing evidence has shown that posttranslational modifications of Notch play important roles in the regulation of the pathway, ranging from glycosylation for the correct maturation of the protein and ligand–receptor interactions (Harvey et al. 2016; Kakuda and Haltiwanger 2017), ubiquitination-dependent regulation of Notch endocytic trafficking and degradation (Shimizu et al. 2014; Steinbuck et al. 2018), to phosphorylation regulating NICD turnover (Fryer et al. 2004; Carrieri et al. 2019). Notch versatile function and regulation have deep implication in physiological processes and diseases.

Functional Roles of Notch in Cancer and How to Target Them

The first proof of a link between Notch pathway and cancer was provided by the identification of Notch mutants in T-ALL and breast cancer (Ellisen et al. 1991; Gallahan and Callahan 1997).

Up to date, the list of cancer-associated Notch mutations has grown together with the number of functions of Notch in cancer. In the past years, studies of Notch in cancers have highlighted its role in tumor growth, cancer-stem like cells, and metastasis, and now Notch role has expanded to metabolism regulation, microenvironment, and tumor immunity. Because of its extensive involvement in cancer, Notch could be a promising target for anticancer therapies. However, its pleiotropic nature poses some challenges.

In recent years, the high-throughput sequencing of tumors has provided a lot of data about the mutational landscape of different tumors, aiming at identifying the most suitable targeting therapies for selected patients. A recent trial, named MOSCATO trial (NCT01566019), aimed to identify genetic alterations in a group of patients with advanced stage malignancies and treat these patients with targeted therapies against the altered pathways identified, including Notch. Although the beneficial outcome was observed in only 7% of the total patients screened and in 24% of patients treated with Notch-targeted therapy (Massard et al. 2017), the results were encouraging and showed that certain patients may benefit from the therapy selection based on genomic landscape. These studies might be greatly improved by a mechanistic investigation of Notch in tumors. For this purpose, understanding Notch status (whether Notch is activated or not) and role in individual tumors/patients will be key to identify tumor/patient responders to selected Notch-targeting therapies.

As we mentioned, Notch can either be an oncogene or a tumor suppressor gene depending on the cell type and tissue in which is expressed. This adds more complexity to Notch targeting in cancer because Notch will have to be either inhibited or activated depending on Notch role in the tissue in which the tumor originated and the cell type we wish to target. Usually Notch is predicted to be tumor suppressive or oncogenic, depending on the role of Notch in the tissue in which the tumor originated; however, this approach might be imprecise. Notch loss-of-function mutations and the suppressive effect of Notch ectopic expression in small cell lung cancers (SCLCs) suggest that Notch acts as a tumor suppressor in these tumors (George et al. 2015). However, an elegant work by Julien Sage's research group showed that Notch is activated and is oncogenic in a subset of tumor cells (10–50%) in SCLC mouse models and human tumors. These cells showed a non-neuroendocrine (non-NE) phenotype, were slow growing, more chemoresistant, and supported the growth of the neuroendocrine (NE) tumor cells (Lim et al. 2017). The activation of Notch in non-NE cells might result from the expression of Notch ligands on NE cells and from the tumor microenvironment (Lim et al. 2017), suggesting that the tumor itself and its microenvironment trigger the generation of non-NE oncogenic-Notch cells. These findings provide a strong rationale for the use of Notch inhibition in combination with other therapies in selected SCLCs or certain stages of the disease where non-NE are found. A similar study showed that subpopulations of cancer stem-like cells with different Notch activation status and different metabolic profiles coexist in glioblastoma (Bayin et al. 2017). These works emphasize the importance of identifying Notch status in tumors to choose the most suitable treatment. For this purpose, the use of biomarkers for Notch signaling activation might be of great help. Not only Notch target genes, but also protein/factors produced by Notch-expressing tumor cells could be used as Notch biomarkers upon identification.

Notch mutations found in cancer are assumed to be loss or gain of function depending on whether Notch is tumor suppressor or oncogenic, respectively, in the tissue in which the tumor originated. However, the outcome of Notch mutations should be validated in order to choose whether the therapeutic intervention should inhibit or favor Notch activation. For example, Notch-targeting therapies could be applied to head and neck squamous cell carcinomas (HNSCCs), since a remarkable number of mutations in Notch genes have been found in HNSCCs in both the Caucasian (10–15%) and Asian populations (50%) (Izumchenko et al. 2014; Song et al. 2014; Agrawal et al. 2011; Stransky et al. 2011). However, it is not clear whether these mutations are gain or loss of function, thus whether Notch should be inhibited

or promoted. In the Caucasian population, the majority of Notch mutations were predicted to be loss of function, whereas in the Asian population mainly gain of function. This classification was ruled out depending on the position of the mutations in the Notch receptor: The Caucasian mutations were mainly clustering around the ligand-binding domain of Notch or causing the truncation of the Ankyrin domain, which is critical for transcription of target genes (Agrawal et al. 2011; Stransky et al. 2011); the Asian mutations were mainly in the Abruptex domain and in the NRR (Izumchenko et al. 2014; Song et al. 2014). There are contradictory evidences on whether Notch is tumor suppressor or oncogenic in HNCCs, and thus a careful functional validation will be needed to determine which of these mutants upregulate and which ones downregulate Notch. Also, these studies point out that the role of Notch might vary not only in different tumor types, but also in tumors of the same class and in different ethnicities. Therefore, a patient-based mechanistic-based use of Notch-targeting therapies is much need.

It is clear that, due to the pleiotropic nature of Notch pathway, the role and status of Notch signaling will have to be evaluated on an individual tumor and patient basis. In particular, this could be achieved with functional/mechanistic studies and identification of biomarkers. This approach will require a considerable amount of effort, but it should pay off in improving the use of Notch-targeting therapies. In the next section, we will discuss current and future Notch-targeting therapies, their mechanistic implications and rationale.

Gamma-Secretase Inhibitors: Learning from Failure

In the past years, γ-secretase inhibitors (GSIs) represented a major class of Notch-targeting agents. GSIs prevent Notch activation by hampering the γ-secretase-mediated cleavage of Notch and the release of NICD. Given the important role of Notch in cancer, GSIs held a lot of expectations in their potential to target cancer cells and especially cancer stem-like cells. Despite the fact that GSIs are the first Notch-targeting agents that saw transition to the clinic, their early-stage clinical development as single agents was challenged by low antitumor effects and severe side effects, due to Notch inhibition in healthy tissues. These included gastrointestinal toxicity, skin rushes, and immunosuppression. Several clinical trials using GSI were terminated or withdrawn before completion. Few trials reported a moderate success. For example, GSI PF-03084014 showed high tolerability, long-term control of the disease, and partial response in 71.4% of patients with desmoid tumors in a phase II clinical trial (Kummar et al. 2017; Villalobos et al. 2018). LY3039478 recently entered clinical trials after proving significant single-agent activity and manageable toxicity in preclinical models (Bender et al. 2013). This agent showed limited therapeutic success in a phase I trial, but with manageable toxicity (Massard et al. 2018). This study, as others with GSIs, was carried out in a heterogeneous cohort of patients with different types of tumors. It is likely that GSIs with improved toxicity, like LY3039478, might be more effective in a selected and validated group of patients. The identification of GSI-responder patients should be one of the major focuses to improve GSI clinical development. An interesting study from O'Rourke and colleagues has identified and validated a transcriptional signature which can predict GSI responders among patients affected by cholangiocarcinomas (CCAs) (O'Rourke et al. 2020). In this study, they first identified an increase in *Notch1*, *Notch3*, and Notch ligands in CCA patients using genomic data, thus rationalizing the potential therapeutic use of GSIs for CCA. Second, they identified a transcriptome signature by treating different CCA cell lines with GSIs, transplanting them in mice and evaluate their sensitivity or resistance to GSI treatment. This signature was then validated in a CCA mouse model and in an independent cohort of CCA patients, in which it identified 48.7% as predicted GSI-responder patients. A similar method could be applied to other cancer types to predict the subgroup of GSI-responder patients and could be very advantageous for the design of clinical trials involving GSIs. The identification

of gene signatures that confirm Notch pathway inhibition would also help establishing and monitoring the therapeutic window of GSIs. A study examined the transcriptome in hair follicle and blood of healthy human and nonhuman primates subministered with GSIs and identified a signature, which correlates with GSI kinetics (Tanis et al. 2016).

It is now clear that GSIs are pharmacologically distinct. GSIs like AL101 (formerly known as BMS-906024) show equal potency in inhibiting all four Notch receptors (Gavai et al. 2015). However, other compounds are more selective toward one or more Notch receptors; PF-3084014 have a stronger effect on Notch2 and, interestingly, a higher potency for Notch3 inhibition at low concentrations (Ran et al. 2017); LY3039478 is a highly potent inhibitor of Notch1 γ-secretase-mediated cleavage (Bender et al. 2013). Given that the roles of the four Notch receptors vary considerably in different cancers, choosing the right inhibitor and dosage to target Notch might improve the therapeutic effect of GSIs. It is now been evaluated the use of GSIs which are selective toward γ-secretase subunits. MRK-560 mainly targets presenilin-1 subunit (PSEN1) in the γ-secretase complex (Borgegård et al. 2012). This compound showed antitumor activity in T-ALL cell lines and patient-derived xenografts and did not cause any major effect in normal T-cells or in the gastrointestinal tract (Habets et al. 2019). This is likely because PSEN1 is highly expressed in T-ALL cells compared to normal T-cells and the lack of PSEN1 might be compensated by the other γ-secretase subunits, thus maintaining tissue homeostasis in the gastrointestinal tract (Habets et al. 2019). The use of more selective GSIs toward Notch or γ-secretase subunits in rationally selected tumors might greatly impact on lowering the toxicity and boosting GSIs anti-tumor activity.

Because γ-secretase have many substrates, GSIs have multiple targets apart from Notch (Haapasalo and Kovacs 2011). On one side, these "off-targets" might contribute to the toxicity associated with these agents, but on the other side, these might also contribute to GSIs antitumor activity. For example, among γ-secretase substrates we find CD44, a cancer stem cell marker, and E-cadherin, which are both associated with tumor progression and invasion (McAuliffe et al. 2012; Marambaud et al. 2002). E-cadherin processing by γ-secretase also increases the amount of free cytosolic β-catenin which is an important mediator of WNT signaling (Marambaud et al. 2002). It is possible that the antitumor effects of PF-03084014 observed in desmoid tumors, which are WNT pathway dependent, might result not only from the inhibition of Notch, but also from the "off-target" effect on WNT-pathway. On the same line, Morgan and colleagues showed that AL101 can enhance the effect of chemotherapy in preclinical models of non-small cell lung cancers (NSCLC), which did not harbor mutations in Notch nor in its negative regulators (Morgan et al. 2017), suggesting GSI antitumor effect does not only depend on Notch inhibition in these tumors. Therefore, upon identification and validation, the broad spectrum of GSIs might be useful to reach multiple targets, including Notch, that drive selected tumors.

As we discussed, GSIs showed a limited activity as single agents in early clinical trials; however, more clinical trials are ongoing with GSIs in combination with chemotherapy or targeted agents. A number of preclinical studies have showed GSIs to enhance the antitumor effect of other anticancer therapies (Morgan et al. 2017; Pikman et al. 2017; Zhao et al. 2016a; Qiu et al. 2013; Schott et al. 2013). Therefore, it is possible that some of these combinatorial treatments might show positive results and allow the use of lower doses of GSIs, thus reducing their side effects.

In summary, even though the clinical development of GSIs has been challenging, the accumulated knowledge about these compounds provides chances for improvements. GSIs are more effective in selected tumors, like desmoid tumors, and it might be possible to identify GSI-responder patients depending on molecular signatures detected in tumors. Also, GSIs that are more selective toward certain Notch receptors might be used to target Notch receptors that are prevalently altered in selected tumors. The "off targets" of GSIs might be exploited to enhance their antitumor effect. GSIs that target specific subunits of

γ-secretase are now available and might reduce the side effects of the classical GSIs. Finally, combinatorial treatments might benefit the antitumor effect of GSIs. Therefore, a more rational use of GSIs should take into account the tumor type, patient responders, Notch alterations, "off targets," and potential combinatorial treatments.

Therapeutic Antibodies Against Notch and Ligands

The use of antibodies to target Notch have advantages compared to pan-Notch inhibition, including higher specificity toward the target. However, preclinical and clinical studies on antibodies targeting Notch and ligands raised some concerns about their use because of toxicity and low antitumor activity. Tarextumab, an antagonistic antibody against Notch2/3, showed promising results in preclinical studies and in a phase I clinical trial in SCLC (Yen et al. 2015), but did not show any benefit in phase II (NCT01859741). The same was for a Notch1 antagonistic antibody (NCT01778439), which also showed severe adverse effects. Anti-DLL4 antibodies were designed to disrupt DLL4–Notch1 interaction and showed to inhibit tumor angiogenesis and growth in preclinical studies (Ridgway et al. 2006; Yan 2011). However, in a phase I clinical trial anti-DLL4 showed dose-limiting adverse toxicities (Chiorean et al. 2015). Nevertheless, anti-Notch2/3 and anti-Notch1 showed a better response in patients with higher expression of Notch3 and Notch1, respectively, and anti-DLL4 showed a partial response in a subgroup of non-small cell lung cancers harboring a β-catenin mutation and in ovarian cancers in which DLL4 is overexpressed (Yen et al. 2015; Chiorean et al. 2015), suggesting that they might be more responsive in a selected patient cohort. The toxicity associated with these antibodies might result from their long half-life in the body, which can result in chronic inhibition of Notch (Yan 2011). Coach and colleagues designed an anti-DLL4, which is rapidly cleared from the body and showed that intermittent inhibition of DLL4/Notch1 mitigates the toxicities associated with continuous inhibition (Couch et al. 2016). As for GSIs, combinatorial use of these antibodies and other therapeutic agents might allow the use of a lower dose, which might decrease toxicity. On the same line, bispecific antibodies able to target multiple hits were recently designed and these might increase the antitumor effect derived from targeting of multiple pathways and also allow the use of lower doses. In a recent study, bispecific antibodies which are able to target both EGFR and Notch2/3 demonstrated anti-tumor effect decreased the number of cancer stem-like cells and presented no major toxicity in cell lines and xenografts models of triple negative breast cancer (Fu et al. 2019). Bispecific antibodies were also designed against DLL4/VEGF and demonstrated inhibition of tumor progression and angiogenesis in xenografts models of lung, breast, and gastric cancers (Lee et al. 2016; Zhou et al. 2019). These antibodies are now moving on to the clinic and showed manageable toxicity and antitumor activity in different previously treated tumors, and especially in ovarian cancer, in a phase I trial (Jimeno et al. 2019). Future clinical investigation should focus on antibody kinetic, multitargeting, and mechanism-based selection of patients to improve anti-Notch pathway antibodies.

Targeting the Sweet Side of Notch

Notch receptors' affinity for different ligands varies and has important regulatory implications on Notch function. Increasing evidence has shown that discrimination and specificity of Notch binding to different ligands rely on differences in glycosylation, binding forces and surfaces, and lipid-binding. Glycosylation is important for Notch–ligand interaction and proper transport of Notch to the cell membrane. Glycosylation in different EGF repeats of Notch receptors has been found to modify the ability of Notch to bind its ligands in *Drosophila* and mammals and is mediated by addition of O-fucose, O-GlcNAc, or O-glucose by the glycotransferases Pofut1, Fringe (Fringe in *Drosophila* and Lunatic, Maniac and Radical Fringe in mammals) and Poglut1/Rumi (Harvey et al. 2016;

Kakuda and Haltiwanger 2017). Pofut1 adds O-fucose and Poglut1 adds O-glucose on specific residues of Notch EGFs. Fringe proteins can extend O-fucose sites by addition of GlcNAc. Glycosylation have different outcomes on receptor–ligand interactions, depending on which kind of sugar is added and which residue is modified (Harvey et al. 2016; Kakuda and Haltiwanger 2017). A comprehensive map of glycosylated site in Notch receptors and structural studies on Notch–ligands interactions identified glycosylated residues of Notch and confirmed their key role in establishing the interaction with ligands (Harvey et al. 2016; Kakuda and Haltiwanger 2017; Luca et al. 2015, 2017). Hartiwanger's group recently developed O-fucose analogs that are incorporated by Pofut1 in Notch1 and inhibit its interaction with DLL1 and DLL4, but not JAG1. This is because Pofut1 adds the analogs, instead of physiological O-fucose, to residues that are important for Notch–ligand interaction (Schneider et al. 2018). The analogs inhibited Notch1 signaling in mammalian cells, zebrafish, and blocked Notch-dependent differentiation of T-cells (Schneider et al. 2018). The potent Notch inhibitory activity and especially their selectivity toward specific ligands make O-fucose analogs appealing for therapeutic intervention. Also, these specific analogs were designed in such a way that they do not affect the physiological biosynthesis of fucose, which is instead affected by other analogs (Schneider et al. 2018). Glycosylation also plays a role in the correct maturation and transport of Notch to the cell membrane. Depletion of *Pofut1* was found to suppress the constitutive activation of certain Notch1 mutants in T-ALL cell lines by reducing the transport of Notch1 to the cell membrane (McMillan et al. 2017). Similar results were obtained by depletion of *Pofut1* in Kras-dependent myeloid leukemia cells and mouse models (Kong et al. 2019). Other agents, like inhibitors of sarco/endoplasmic reticulum calcium ATPase (SERCA) or heat shock protein 90 (Hsp90), which block the correct maturation of Notch in the ER, showed similar results in T-ALL cell lines and mouse models (Roti et al. 2013; Wang et al. 2017). Interestingly, these agents inhibited Notch without severe toxicity in mouse models. It is possible that because of their structural defects certain Notch mutants are more sensitive to impaired maturation compared to wild type. Therefore, inhibition of Pofut1, SERCA, or Hsp90 might allow a more specific targeting of selected Notch mutants.

Recent work on the structural resolution of Notch1 receptor and its ligands DLL4 and JAG1 showed that different regions of Notch extracellular domain are required for the interaction with different ligands. DLL4 mainly interacts with Notch1 EGF 11–12, whereas EGF 8–10 also significantly contributes to the interaction with JAG1 (Luca et al. 2015, 2017). Further, measurement of forces in Notch–ligand interaction showed that DLL4 and JAG1 require different tension forces in their binding to Notch1 (Luca et al. 2017). Recent studies also showed that the N-terminal region of Notch ligands can interact with lipids present on the cell membrane of the Notch-expressing cells (Kershaw et al. 2015; Suckling et al. 2017). Interestingly, Notch ligands lipid-binding preference varies and might represent another regulatory mechanism for specific Notch–ligand interaction (Suckling et al. 2017; Shilo and Sprinzak 2017). Given that the lipid composition of the cell membrane is heterogeneous, the position of Notch in different subdomains of the cell membrane might also affect its interaction with ligands. These findings have profound implications in the design of Notch-targeting therapies. Antibodies against Notch and ligands or engineered Notch receptors and ligands are currently under development and will have to carefully take the requirements for Notch–ligand interaction into account to make these functional and specific.

Notch in the Endocytic Maze

An increasing number of evidences showed that ubiquitination of Notch mediated by different ubiquitin ligases orchestrates the degradation and the ligand-independent activation of Notch. This process involves the endocytosis of Notch receptor and its sorting in different endocytic

compartments (Shimizu et al. 2014; Steinbuck et al. 2018; Wilkin et al. 2008; Alfred and Vaccari 2018; Yamada et al. 2011; Hori et al. 2011; Schneider et al. 2013). This mechanism has mainly been described in *Drosophila*, however the evidence of a similar regulation in mammals and its relevance in cancers is increasing. The amount of full-length Notch receptors at the cell membrane could be regulated through lysosomal-dependent degradation. This process seems to be mediated by the *Drosophila* HECT E3 ubiquitin ligase, suppressor of Deltex (Su(dx)), and its mammalian ortholog Itch/AIP4 since both were found to poly-ubiquitinate the intracellular domain of Notch and lead the receptor to endosomal internalization and lysosomal-dependent degradation (Shimizu et al. 2014; Wilkin et al. 2004; Chastagner et al. 2008; Yao et al. 2018). Other ubiquitin ligases, including c-Cbl and Nedd4, were also showed to have analogous functions (Wilkin et al. 2004; Jehn et al. 2002; Platonova et al. 2015). It is not completely clear whether this negative regulatory machine directly induces the endocytosis of Notch from the cell membrane or diverts Notch from a constitutive recycling route or other endocytic pathways (Shimizu et al. 2014; Wilkin et al. 2004). Depletion of components of the *Drosophila* recycling retromer machinery was found to cause accumulation of Notch in endosomes and ectopic ligand-independent activation (Gomez-Lamarca et al. 2015), suggesting recycling and endocytic degradation of Notch might be linked and both contribute to Notch turnover. Numb, a conserved adaptor protein, also plays a role in regulating Notch endocytic degradation, likely by facilitating the interaction between Itch/AIP4 and Notch (McGill et al. 2009). Interestingly, Numb was found downregulated in breast cancer cell lines and primary breast tumor cells leading to increased Notch activation (Pece et al. 2004; Stylianou et al. 2006), thus confirming the importance of this degradative regulatory mechanism. Recently, it was showed that Numb overexpression reduces metastasis and tumor growth in breast cancer mouse models (Zhang et al. 2016). Another recent finding showed that Vasorin, a protein frequently overexpressed in glioma stem-like cells in hypoxic conditions, blocks Numb-dependent degradation and stabilizes Notch at the cell membrane for activation (Man et al. 2018). Importantly, silencing of Vasorin reduced Notch and tumor growth in glioblastoma mouse models (Man et al. 2018). Similarly, inhibition of PI3K-AKT was found to cause the lysosomal degradation of Notch upon ubiquitination by c-Cbl in T-ALL cells (Platonova et al. 2015). These recent findings suggest that the lysosomal-dependent degradation of Notch is conserved and is an important regulatory mechanism for the homeostasis of Notch pathway in different cellular contexts. Targeting Notch degradation might represent a strategy to inhibit or reactivate Notch.

Different studies in *Drosophila* have reported that endocytosis and ubiquitination can not only lead to Notch degradation but also to activation in the endosomes. In *Drosophila*, Deltex, a ring-finger ubiquitin ligase, was found to mono-ubiquitinate Notch and sort it for lysosomal-dependent proteolytic activation (Shimizu et al. 2014; Wilkin et al. 2008; Yamada et al. 2011; Hori et al. 2011). In this way, Dx competes with Su(dx) for the endocytic sorting of Notch (Shimizu et al. 2014; Wilkin et al. 2008). This form of endosomal activation is ligand-independent and requires γ-secretase cleavage, but not S2 cleavage (Shimizu et al. 2014; Wilkin et al. 2008; Gupta-Rossi et al. 2004), which might be bypassed thanks to the acidic ionic environment or lysozymes present in the lumen of endosomes which could unmask and cleave S2 site (Steinbuck et al. 2018; Vaccari et al. 2010; Kobia et al. 2014). This was also supported by the observation that genetic and pharmacological inhibition of the vacuolar H+ ATPase, which is responsible of the acidification of endosomes, reduces Notch endocytic activation in *Drosophila* tissues and mammalian breast cancer cell lines (Vaccari et al. 2010, 2008; Kobia et al. 2014; Faronato et al. 2015). Dx has five mammalian orthologs of which three can bind to Notch, such as DTX1, DTX2, and DTX4 (Matsuno et al. 1998; Chastagner et al. 2017). Old literature showed that mammalian Dx proteins act either as positive

or negative regulators of Notch in different contexts (Matsuno et al. 1998; Sestan et al. 1999; Yamamoto et al. 2001; Izon et al. 2002). An interesting recent work showed that DTX4 enhances ligand-dependent activation of Notch1 by favoring its endocytosis and S2 cleavage (Chastagner et al. 2017). Dx role needs further investigation; however, it is possible that different mammalian Dx proteins have distinct regulatory functions and their role might depend on the cellular context or their interactions with other regulators. In *Drosophila* it was found that interaction of Dx with Kurtz (Krz), the ortholog of the human non-visual B-arrestin 2, is critical for the sorting of Notch to endosomal degradation or activation (Hori et al. 2011; Mukherjee et al. 2005). Dx–Krz–Notch complex seems to act as a platform for the recruitment of other regulators (Hori et al. 2011; Schneider et al. 2013; Mukherjee et al. 2005). It is very likely that Su(dx) may also join this complex and Krz acts as a switch between Dx and Su(dx) and in turn, between endosomal degradation and activation. Notch endocytic trafficking is also regulated by Endosomal Sorting Complex Required for Transport (ESCRT). It was showed that *Drosophila ESCRT* mutants, which block different steps of the endocytic trafficking, lead to aberrant ligand-independent activation of Notch (Vaccari et al. 2008). Similarly, knockout of the ESCRT1 component Tumor-susceptibility-gene-101 leads to the endosomal activation of Notch in human cell lines (Leitch et al. 2014). Also, Shrub, a subunit of the ESCRT III complex, was found to contribute to the Dx–Krz–Notch complex (Hori et al. 2011; Schneider et al. 2013). These observations suggest that ESCRTs contribute to the endocytic sorting of Notch, and that defect in the endocytic machinery could lead to uncontrolled ligand-independent Notch signaling, something that could be happening in cancer cells. New regulators of the endocytic trafficking of Notch have been identified in recent years. Cis-inhibition is a known mechanism by which cis-interaction between Notch receptors and ligands expressed in the same cell inhibits Notch signaling (Sprinzak et al. 2010; del Álamo et al. 2011). An elegant work from Wu-Min Deng's group showed that ligand-independent activation of Notch can be inhibited by cis-inhibition in different *Drosophila* tissues in mutant and physiological conditions (Palmer et al. 2014). Similarly, Crumbs (Crb), a conserved large transmembrane protein involved in cell polarization, was found to inhibit Notch ligand-independent activation by blocking Notch at the cell surface in *Drosophila* epithelial wing tissue and Crb depletion leading to upregulation of Notch (Nemetschke and Knust 2016; Das and Knust 2018). Recent work reported for the first time that cis-activation occurs in vitro in mammalians cells in the absence of Notch ligands in trans (Nandagopal 2019). However possible, it is not clear if this process is linked to endocytic regulation of Notch.

The physiological function of the endocytic regulation of Notch is not fully understood, but intriguing hypotheses are rising. It is possible that this mechanism acts as a regulatory network that tunes the ligand-dependent activation against different environmental changes and stress conditions, since this was found to regulate the amount of ligand-dependent signaling in *Drosophila* in response to temperature variation (Shimizu et al. 2014). For instance, hypoxia and nutrients availability might represent stress conditions in which the endocytic regulation ensures Notch signaling homeostasis in mammals. This mechanism could also control Notch signaling in contexts in which ligand binding is challenging. This could be the case of circulating cells as hemocytes and lymphocytes. Indeed, recent works suggest that the activation of Notch in CD4+ and CD8+ T-cells is ligand-independent and likely linked to endosomal regulation (Sorrentino et al. 2019; Steinbuck and Winandy 2018). It is also very interesting that depletion of different endocytic components leads to uncontrolled activation of Notch, which might be relevant to aberrant Notch signaling in diseases. Tuberous sclerosis, a dominant genetic disease which causes the growth of benign tumors, is caused by mutations in *Tuberous Sclerosis* 1 and 2 (TSC1 and 2) and characterized by upregulation of Notch. TSC1 and 2 are lysosomal-associated regulators that

were first believed to regulate mTOR; however, recent studies suggest that TSC1 and 2 might be direct regulators of Notch (Ma et al. 2010; Karbowniczek et al. 2010; Cho et al. 2017), and it is possible this involves endosomal regulation. These mechanisms have been mainly described in *Drosophila*; however, it would be interesting and significant to further explore these mechanisms in the mammalian system and in diseases. Notch is a highly conserved pathway and is likely that similar mechanisms are found in mammals and might help to elucidate Notch regulation in physiological and, importantly, in disease conditions.

Exosomes and other types of extracellular vesicles have received great attention in recent years because of their capacity to transfer signaling molecules between cells. Notch1 together with γ-secretase was found in exosomes secreted by *Tsc1*-null cells (Patel et al. 2016). Notch1 containing exosomes were delivered to healthy cells where the transported Notch was activated leading to the acquisition of a *Tsc1*-like phenotype in recipient cells (Patel et al. 2016). Contrarily, ligands were also found to be transported via exosomes and to cis-inhibit Notch in the recipient cells (Sheldon et al. 2010). Since exosomes originate from late endosomes, a link between the endocytic trafficking of Notch and its transport into exosomes is possible. Similarly, Notch2 was found in ARMM vesicles (arrestin domain-containing protein 1–mediated microvesicles), which buds from the cell membrane, and to be transported and activated in recipient cells (Wang and Lu 2017). Interestingly, Itch and ADAM10 were involved in the loading of Notch to ARMMs and were also incorporated in the vesicles (Wang and Lu 2017). Therefore, Notch might also deliver its signaling in nonadjacent cells via extracellular vesicles. Notch signaling in recipient cells is likely to be ligand independent.

Endocytic trafficking of Notch seems to tightly regulate Notch homeostasis and deletion of endocytic regulators, leading to uncontrolled Notch signaling. A better understanding of Notch endocytic regulation might reveal mechanisms by which Notch is deregulated in cancer and provide new means for Notch-targeting therapies. The endocytic regulation of Notch can lead either to activation or degradation of Notch; therefore targeting this regulation might provide ways to inhibit or enhance Notch signaling.

Notch as a Metabolic Reprogrammer

Metabolism reprogramming is now considered a major hallmark of cancer, through which cancer cell can adapt and survive to different environmental changes, develop resistance to treatments, and modulate antitumor immunity. These mechanisms are tightly entangled with Notch. Metabolic reprogramming mediated by Notch has been reported in different hematological (Kong et al. 2019; Jitschin et al. 2015; Kishton et al. 2016). and solid tumors (Bayin et al. 2017; Bhola et al. 2016)

In physiological conditions, Notch regulates cell size, glucose uptake, and glycolysis through activation of PI3K/Akt or directly by transcriptional regulation of metabolic genes, including c-Myc (Ciofani and Zúñiga-Pflücker 2005; Wang et al. 2011). Interestingly, more recent evidence showed that Notch can reprogram metabolism by direct transcriptional regulation of mitochondrial DNA. It was observed that NICD is recruited to mitochondrial DNA and upregulates respiratory chain components to favor pro-inflammatory activation of macrophages (Xu et al. 2015). Also, mitochondrial metabolism seems important to sustain cancer cells and Notch might be linked to it (Kong et al. 2019; Herranz et al. 2015). Up to date, Notch-dependent metabolic regulation has been reported to sustain survival of T-cell progenitors, CD4+ memory T-cells, and activation of macrophages (Ciofani and Zúñiga-Pflücker 2005; Xu et al. 2015; Maekawa et al. 2015), and might be involved in the metabolic regulation of other immune cells, given Notch's important role in immunity. Several studies showed that tumors counteract T-cells antitumor responses by hampering T-cells glycolytic metabolism (Molon et al. 2016). It is not yet known how this is achieved, but recent reports showed this might be

via Notch downregulation in T-cells (Zhao et al. 2016b). Further studies are needed to confirm this link, but targeting T-cell metabolism or Notch might represent a way to counteract tumor-mediated immunosuppression.

Metabolism reprogramming seems responsible for the development of cancer cell resistance to therapies. Therapeutic inhibition of Notch in T-ALL leads to reduction of glutamine usage, which should hamper T-ALL survival (Herranz et al. 2015). However, in response to Notch inhibition, T-ALL cells activate autophagy for the recovery of nutrients to sustain their metabolism, possibly leading to resistance. This resistant mechanism can be counteracted by inhibition of glutaminolysis and autophagy, since this was showed to increase efficacy of Notch inhibition in T-ALL (Herranz et al. 2015). Resistance to therapeutic inhibition of PI3k/mTOR, often observed in triple-negative breast cancers, was found to be caused by activation of mitochondrial metabolism via Notch1 (Bhola et al. 2016). Pharmacological inhibition of Notch reduced tumor formation and resistance in triple-negative breast cancer xenografts (Bhola et al. 2016).

Differences in the metabolic profile of cancer cells versus healthy cells might be critical to design targeting strategies that affect cancer cell metabolism and spare normal cells. Both normal T-cells and T-ALL were thought to rely on aerobic glycolysis promoted by PI3K and c-Myc (Ciofani and Zúñiga-Pflücker 2005; Palomero et al. 2007). However, analysis of primary T-ALL and normal T-cells showed that their metabolism is different and this is because of Notch. In T-ALL, Notch promotes glycolysis, but also induces activation of AMPK, which favors mitochondrial metabolism over glycolysis, which seems to promote T-ALL survival (Kishton et al. 2016).

Tumor microenvironment influences metabolism reprogramming and heterogeneity. Stroma cells were found to promote glycolysis and survival in B-cell chronic lymphocytic leukemia via activation of Notch and its transcription target c-Myc (Jitschin et al. 2015). Differential activation of Notch was found to regulate the metabolic status in glioblastoma stem cell subpopulations (Bayin et al. 2017). In this study, cells with high activation of Notch relied on aerobic glycolysis and resided in vascular microenvironment, whereas cells with low Notch depended on anaerobic metabolism and resided in hypoxic microenvironment. Importantly, reactivation of Notch in the second group of cells reversed their metabolism from anaerobic to aerobic and abolished their resistance to hypoxia (Bayin et al. 2017).

A role for metabolism in cancer has been known since early studies; however, this has gained attention and been explored only in recent times. Further investigation will be needed to crack down metabolism reprogramming in cancer and its link with Notch. Nevertheless, current evidence provides a rationale for Notch/metabolism-targeting to increase antitumor immunity, counteract therapy resistance, and adaptation of cancer cells.

Notch for Immunotherapy

Accumulating evidence has shown that Notch is heavily involved in shaping the immune system in physiological conditions and the pro-tumoral immune microenvironment in cancer (Grazioli et al. 2017; Hossain et al. 2018). Together with the rising enthusiasm for the use of immunotherapy for cancer, this provided a strong rationale for the evaluation of Notch-targeting strategies as immunomodulators and opened up a new research direction in the Notch-in-cancer field, which previously mainly focused on targeting stem-like and bulk tumor cells.

Notch is crucial in the development and maintenance of different immune cells both in the adaptive, specific, and long-lasting as well as innate, fast, and unspecific immunity. Notch determines the specification and lineage of adaptive T-cells CD4+, CD8+, and Natural killer cells in the thymus and the survival, function, and differentiation to memory lineage of peripheral T-cells. At the same time, Notch also regulates the differentiation of innate immune myeloid cells (granulocytes, macrophages, dendritic cells [DCs]), and crosstalks between myeloid cells

and T-cells during immune responses. Some of these processes, including differentiation of T-cells and crosstalks between immune cells, are mediated by Notch ligands. For example, it was showed that the expression of different ligands in DCs stimulates the differentiation of CD4+ T-cells into different lineages during immune responses (Kassner et al. 2010; Biktasova et al. 2015; Meng et al. 2016). However, other processes might rely on ligand-independent Notch signaling. It was recently showed that Notch activation in CD4+ T-cells is ligand-independent and involved Notch endocytosis (Steinbuck et al. 2018). This form of activation is triggered by stimulation of T-cell receptor (TCR)/CD28 receptor and PI3K pathway followed by downstream events that facilitate the proteolytic cleavage of Notch (Steinbuck et al. 2018).

In the tumor microenvironment, protumor and antitumor immune cells coexist and antagonize each other. Notch is important for both protumor and antitumor immunity and for their crosstalk. CD4+ T-helper 1 and CD8+ cytotoxic T-cells are the main weapons of our immune system against cancer because they can recognize and induce cell death in malignant cells. Unfortunately, tumors are very skilled in evading our body immune response by different means: immunosuppressive molecules, inhibitory ligands, and suppressive cell types. Different studies showed that Notch is decreased in tumor-infiltrating T-cells, and reactivation of Notch enhances antitumor immunity in mouse models (Sierra et al. 2014; Huang et al. 2011; Sugimoto et al. 2010). In particular, a pivotal work by Paulo Rodriguez's research group demonstrated that Notch1 and Notch2 are downregulated in tumor-infiltrating CD8+ T-cells and, strikingly, ectopic expression of Notch1 NICD in CD8+ T-cells enhanced their cytotoxic response and antitumor activity in vivo in mouse models (Sierra et al. 2014). These findings suggest that Notch is targeted by tumor-mediated immunosuppression and let to the idea that reactivation of Notch in T-cells might protect them from the tumor-mediated immunosuppression and boost their antitumoral activity. Therefore, Notch-targeting therapies are worth exploring for immunotherapy.

Bortezomib, a FDA-approved proteasome inhibitor for multiple myeloma, mantle cell lymphoma, and NSCL cancer, was found to favor antitumor immunity by rescuing Notch1 and Notch2 in CD8+ cells from the tumor-mediated immune suppression and enhance the production of effectors and stimulatory cytokines (Thounaojam et al. 2015; Pellom et al. 2017). These findings led Shanken and colleagues to apply bortezomib for adoptive T-cell therapy. They successfully showed that treatment with bortezomib-sustained T-cell function after transfer of the treated T-cells in the host mice and reduced tumor burden in human renal carcinomas xenografts (Shanker et al. 2015). Despite the success of this study there have been no further advances in this direction. Only low doses of bortezomib seems to elicit a positive effect on immune cells, while high doses were reported to suppress immune cells (Berges et al. 2008), suggesting that the effect of proteosomal degradation inhibition on Notch pathway in T-cells might be complex and needs further investigation. Indeed, it is not yet clear how bortezomib have an impact on T-cells. Some studies reported that this regulation might rely on the crosstalk between NICD and Nuclear Factor kB (NFkB), which together can enhance CD8+ effector function (Thounaojam et al. 2015), or to positively regulate miR155, the suppression of which seems to downregulate Notch in T-cells (American Association of Immunologists 2018, 2019). Given that Notch turnover, which is mediated by proteosomal and lysosomal degradation, is key to ensure the fine regulation of Notch, it is also possible that bortezomib might rescue Notch receptor or one of its regulators from proteosomal degradation, thus increasing Notch activation in T-cells. Further mechanistic description of bortezomib-dependent Notch modulation will be needed for the safe use of this agent for immunomodulation.

Another way in which tumors suppress the immune response is through the production of adenosine in the tumor microenvironment. This molecule stimulates the adrenergic receptors A2AR, A2BR, A1R, and A3R and have different regulatory effects depending on the receptor and

the cell in which it is expressed (Vijayan et al. 2017; Leone and Emens 2018). Adenosine was found to have a direct suppressive effect on CD8+ through the stimulation of the adenosine receptor A2AR (Ohta et al. 2006). Conversely, several studies have shown that genetic or pharmacological inhibition of A2AR, using A2AR antagonists, restores antitumor immunity and counteracts adenosine-mediated immunosuppression (Waickman et al. 2012; Beavis et al. 2013a, b). These compounds also showed to enhance the effect of checkpoint inhibitors (PD1, PDL1, and CTL4) in preclinical mouse models and a number of A2AR antagonists are now in clinical development (Willingham et al. 2018; Iannone et al. 2014; Mittal et al. 2014; Beavis et al. 2015). Also, A2AR inhibition was found to potentiate the efficacy of adoptive CAR-T cell therapy in HER2+ mouse models, likely because of boosting of T-cell effector function and resistance (Beavis et al. 2017). Morello and Miele's research groups recently showed that stimulation of A2AR inhibits the activation of Notch1 and in turn the production of INF-γ and Granzyme B in CD8+ cells (Sorrentino et al. 2019). Importantly, treatment with an A2AR antagonist restored Notch1 and the effector production, suggesting inhibition of A2AR might enhance CD8+ effector function through Notch (Sorrentino et al. 2019). This is very interesting because it shows that adenosine affect CD8+ effector function via Notch, thus placing Notch at the core of the adenosine-mediated immunoregulation and A2AR antagonists mechanism of action. Also, this study proposed that A2AR-mediated regulation of Notch might involve its endocytic regulation, similarly to what was found in CD4+ T-cells (Steinbuck et al. 2018). Because of their effect on releasing the "brakes" of antitumor immune response, as PD1/PDL1/CTL4, A2AR antagonists have been referred as the "next generation of checkpoint inhibitors." (Leone et al. 2015). In light of their recent link with Notch, A2AR antagonists might turn out to be one of the first examples of Notch-modulating immunotherapy. Further studies on how adenosine receptors regulate Notch will be required to maximize the therapeutic application of adenosine receptor antagonists and avoid unwanted off-target effects. As we discussed, both Notch and A2AR, or more generally adenosine receptors, are expressed in different sets of cells within the tumor microenvironment, and the function of their crosstalk might vary.

Notch is not only involved in the intrinsic properties of T-cells, but also in the crosstalk between T-cells and other regulatory immune cells. In physiological conditions, myeloid cells differentiate in several regulatory immune cell types (macrophages, dendritic cells, granulocytes), which are recruited by inflammation and control the immune response. The tumor microenvironment releases signals that perturb the differentiation of myeloid cells, leading to the generation of myeloid-derived suppressive cells (MDSCs), dendritic cells (DCs), and tumor-associated macrophages (TAMs), which suppress the antitumoral immune response (Hossain et al. 2018). Recent papers showed that Notch ligands play a major role in both the specification of these pro-tumoral immune cells and their crosstalk with T-cells. It was observed that pro-tumoral MDSCs overexpress JAG1 and JAG2 and have a decreased expression of DLL1 and DLL4 (Sierra et al. 2014, 2017). On the contrary, it was showed that expression of DLL1 or DLL4, but not JAG1 or JAG2, in DCs stimulates T-cell effector and memory functions (Kassner et al. 2010; Biktasova et al. 2015; Meng et al. 2016). Also, expression of JAG1 or JAG2 in DCs correlates with PD-1 expression in tumor-infiltrating CD8+ effector and memory T-cells, whereas expression of DLL1 or DLL4 correlates with the expression of Notch receptors (Tchekneva et al. 2019). These observations suggest that JAG1/2 and DLL1/4 generally favor pro-tumoral and anti-tumoral immunity, respectively. Indeed, targeting of Notch ligands had a positive outcome in preclinical mouse models. Systemic administration of JAG1/2 blocking antibodies improved antitumor immune response, inhibited MDSCs, and enhanced adoptive T-cell therapy in lung, colon, melanoma, and thymoma mouse models (Sierra et al. 2017). On the same lines, engineered DLL1 multivalent clustered construct or JAG1 monovalent construct, which stimulates DLL1 signaling and inhibits JAG1 signaling,

respectively, improved antitumor immune response and reduced PD1 expression in pancreatic and lung cancer mouse models (Huang et al. 2011; Tchekneva et al. 2019). The significance of these studies is that targeting Notch ligands might represent a way to modulate the immune response in the tumor microenvironment and the development of antagonistic antibodies or engineered Notch ligands might be an attractive therapeutic strategy.

Notch is also important for the differentiation of MDSCs, DCs, and TAMs in the tumor microenvironment. Anti-JAG1/2 seems to inhibit MDSCs or to induce their switch to a non-immunosuppressive phenotype (Sierra et al. 2017). It is not clear how this is achieved, but it is possible that inhibition of Jagged in MDSCs or adjacent cells ultimately modulate Notch in MDSCs (Sierra et al. 2017). In DCs Notch stimulation positively modulates their response to pro-inflammatory signals (Gentle et al. 2012). Majority of TAMs downregulate Notch and acquire a M2-anti-inflammatory phenotype; however, reactivation of Notch in TAMs favors their M1-pro-inflammatory phenotype and ameliorate antitumor immunity (Xu et al. 2015; Wang et al. 2010).

Targeting Notch demonstrated a remarkable effect on antitumor immunity and has a promising future. Since Notch plays a different role in different cells in the tumor microenvironment, the main challenge of systemic immunomodulation will be to design strategies that selectively target Notch in the desired immune cells. In line with this idea, targeting of ligands seems an attractive strategy to modulate the crosstalk between immune cells in the tumor microenvironment. On the other hand, since Notch seems regulated in a ligand-independent way in T-cells, it might be interesting to explore strategies to selectively target this unique mode of activation. The important role of Notch in immunomodulation also highlights that Notch-targeting therapies directed to cancer and stroma cells will have to be selective enough to not affect Notch in immune cells. For example, pan-Notch GSIs have a demonstrated immunosuppressive activity and this might play in favor of the tumor.

Notch in ACTion

In the era of immunotherapy, adoptive T-cell therapy (ACT) is one of the most exciting T-cell-based technologies and Notch is at the frontline of its development. ACT is based on the in vitro generation of T-cells, which are able to recognize tumor-specific antigens and are then transferred in the patients where they will trigger a potent antitumor immune response (Garber 2018). T-cells for ACT are either generated and instructed in vitro from tumor infiltrating T-cells taken from the patient or are engineered T-cells, which present a transgenic T-cell receptor (TCRs) or a chimeric-antigen receptor (CARs) (Garber 2018). ACT has shown remarkable results in clinical trials in B-ALL and melanoma (Dudley et al. 2008; Besser et al. 2010; Brentjens et al. 2013; Grupp et al. 2013). However, this technology has some limitations, which need to be addressed to expand its use to other tumors and increase its effectiveness and safety. The limits of ACT are the low number of T-cells recovered from the patient, tumor-specific antigen recognition, and immune suppression in the tumor microenvironment. In particular, increasing the number of cells is critical for ACT because only a limited number of T-cells can be isolated from the patient. CARs recognize specific antigens on the surface of cells, while TCRs have a broader recognition potential, because they recognize peptides from antigen-presenting cells. However, both can lose antigen recognition because of change of antigens expressed in tumor cells and suppression of antigen-presenting cells in the tumor microenvironment. Also, this might give rise to unspecific immune responses if the antigen recognition is not cancer-cell specific. Finally, all kinds of ATC, as endogenous T-cells, have to counteract immunosuppression in the tumor microenvironment. Given the direct involvement of Notch signaling in T-cell intrinsic functions, tolerance, and differentiation, Notch modulation is an attractive strategy to address ACT limitations. In the previous chapter we saw that Notch ligands are critical for T-cells maintenance in the tumor microenvironment. To generate a higher number of T-cells for ACT, different groups exploited Notch-induced

differentiation by culturing induced pluripotent stem cells (iPSC) on DLL1-expressing stroma cells (Lei et al. 2011) or, more recently, hemato-vascular mesodermal progenitors on DLL4-expressing stroma cells (Kumar et al. 2019) obtaining a high number of T-cells and increasing their in vitro expansion capacity. Recent studies also tried to generate T-stem memory cells for ACT by coculturing activated T-cells from mouse or humans with DLL1-expressing stroma cells (Kondo et al. 2017). These cells, named iTscm, had features of memory cells, like self-renewal and rapid response to antigens, lower expression of inhibitory ligands (PD1, CTL4), and showed stronger antitumor effect in humanized Epstein–Barr virus transformed-tumor model mice (Kondo et al. 2017, 2018). Importantly, iTscm can be generated from tumor-infiltrating T-cells from the patient, thus overcoming the need of engineered antigen recognition.

Till date, a number of agents has shown the ability to increase T-cell tolerance via Notch signaling against immune suppression and these could be employed to improve ACT resistance. As we mentioned, bortezomib potentiated ACT in human renal carcinomas xenografts (Shanker et al. 2015). Also, it was showed that treatment with an A2AR antagonist increases the activation and effector function of CARs and their efficacy in HER2+ cancer mouse models (Beavis et al. 2017). These compounds represent a potential asset that can be applied to boost ACT resistance against tumoral immune suppression; however, there is no yet evidence that these treatments will enhance ACT in human tumors.

SynNoches: Sin or Miracle?

The mechanism of activation of Notch receptor is fascinating, having the extracellular domain responding to external clues and triggering the release of NICD to deliver intracellular responses. This inspired researchers to build synthetic Notch receptors, called synNotches, which have customizable extracellular and intracellular domains linked by the transmembrane domain of Notch, thus allowing customizable extra- to intracellular signaling. Recently, synNotches have been extensively applied to ACT to improve the antigen recognition of engineered T-cells and for many other applications, such as delivery of drugs or pro-immunity signals in the tumor microenvironment. Wendell Lim and his group were the firsts to design a synNotch receptor, which, upon recognition of a specific antigen, triggers the expression of a CAR in the same T-cell, which recognizes a second antigen (Roybal et al. 2016; Morsut et al. 2016). They showed that these engineered T-cells were able to recognize and kill cancer cells that express both antigens and not only one of them, in vitro and in vivo in mouse models (Roybal et al. 2016). This strategy could improve the efficacy of engineered T-cells especially in solid tumors that do not express a specific antigen, where the recognition of multiple antigens instead of one will greatly increase the chances of targeting. Further, this could avoid the unspecific targeting of healthy cells that express one of the antigens present in cancer cells, which could cause severe side effects. Using synNotch technology, T-cells were also engineered to drive a plethora of other functions, such as delivery of therapeutic molecules (antibodies, cytotoxic proteins, apoptosis inducers) to increase the antitumor effect, pro and suppressive immune signals (cytokines, ligands, master regulators, adjuvants) to regulate the immune response in the tumor microenvironment (Morsut et al. 2016), thus showing the versatility of this technology. SynNotch-CARs have opened up a new platform for molecule targeting and delivery which seems to have almost unlimited possibilities. Several investigators are now using synNotches to establish new therapeutic strategies. ROI is a potential target for CAR therapy since it is expressed in different solid tumors; however this antigen is also expressed on stroma cells, thus arising the possibility of severe toxicity upon ROI targeting. Recently, T-cells were engineered with a synNotch recognizing the tumor antigen EpCam or B7H3, which triggers the expression of a CAR specific for ROI, thus allowing the specific targeting of tumor cells only and sparing of ROI+ stroma cells (Srivastava et al. 2019). Another group designed

synNotch-CAR T-cells which express an antibody fragment against Ax1, an antigen expressed in different tumors, which led to increased cytokine production and targeting of Ax1-expressing tumor cells in mouse models (Cho et al. 2018).

It was also proposed that synNotches could be broadly used to modify the cellular microenvironment in different contexts (Morsut et al. 2016). Since Notch machinery is ubiquitously expressed, this technology could be applied to different cell types. For example, the development and organization of tissues is controlled by cell–cell communication which produce specific morphological signals and Notch is well known to play a role in tissue patterning and morphogenesis. A recent study used synNotches to engineer morphological signals between cells to lead the self-organization of multicellular structures for tissue engineering (Toda et al. 2018), therefore synNotches could be used to customize morphological or reprogramming signals. More generally, synNotches were used to study cell–cell interactions in different *Drosophila* tissues, suggesting this technology might be extensively used to study developmental processes in which cell–cell interactions are critical, including cell competition, differentiation (He et al. 2017), tissue morphogenesis, and tumorigenesis. Other applications of synNotches include platforms to identify and study transmembrane receptors which are activated by proteolysis similarly to Notch (Hayward et al. 2019).

SynNotches applied to T-cells engineering have shown remarkable therapeutic applications with promising clinical perspectives. However, this technology is young and will need further establishment and evaluation before reaching clinical development. Application of synNotches to tissue engineering are also very intriguing, but due to the important involvement of Notch in tissue morphogenesis, safety will have to be carefully addressed. This is valid for all applications that will aim to use synNotches as synthetic modulators in biological processes. On the other hand, SynNotches could be a very powerful tool to study these processes. SynNotches have surely shown to be incredibly versatile and their employment in different technologies can be easily foreseen.

Discussion and Conclusion

Notch is a fascinating signaling pathway. From its discovery in *Drosophila* to Notch entry in cancer immunotherapy, Notch field saw a continuous revolution. However, it seems that we still have not unravel all the secrets and potentials of this pathway. In this chapter, we discussed current and new Notch-targeting therapies with their exciting promises and challenges. Notch pleiotropic nature seems to be both the advantage and the challenge of Notch-targeted therapies. Targeting Notch allows to virtually modulate any aspect of cancer; however, this means that Notch-targeting must be highly specific toward the desired target. This chapter highlighted different factors that are critical to ensure the specificity of Notch-targeting. Due to the complexity of its regulation, Notch can be modulated in many different ways. Understanding the mechanism by which Notch is modulated in different sets of cells within the tumor microenvironment will be crucial to predict whether Notch-targeting therapies will be effective and to identify new druggable targets. Understanding whether Notch function is pro- or anti-tumoral is essential, especially because Notch is differently expressed in subsets of cells within the tumor and the microenvironment. The first Notch-targeted therapies were designed to inhibit Notch; however, it is now becoming clear that in certain situations Notch should be favored instead of inhibited. Recent investigation on Notch regulation has revealed alternative ways in which Notch can be activated or inhibited. For example, the endocytic regulation of Notch lead to either degradation or activation and this might be an attractive mechanism to inhibit or reactivate Notch. Finally, preclinical and clinical trials demonstrated that certain patients/tumors are more responsive to Notch-targeting therapies. Therefore, selection of patient responders and identification of signatures should be implemented for the rational use of Notch-targeting therapies. The new means of Notch-targeting and their applications to new fields hold promising perspectives and it will be exciting to see which advances they will bring to cancer therapy.

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Unfortunately the book was published without correcting a typo in the author name in chapter 8. The author name has been corrected now to read as follows:

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