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Role of Transforming Growth Factor β-regulated microRNA in breast cancer progression

by

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August 2013

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

List of Abbreviations

ALK	*Activin receptor-like kinase*
AP1	*Activator protein 1*
Apaf1	*Apoptosis activating factor-1*
ARTS	*Apoptosis-related protein in the TGF-β signaling pathway*
ASO	*Antisense oligonucleotide*
BC	*Breast cancer*
BL	*Basal-like*
BMP	*Bone morphogenetic protein*
BRCA	*Breast cancer susceptibility protein*
CD	*Cluster of differentiation*
CDK	*Cyclin-dependent kinase*
CLC	*Cardiotrophin-like cytokine*
CNTF	*Ciliary neutrophic factor*
COX2	*Cyclooxygenase-2*
CTGF	*Connective tissue growth factor*
CXCR4	*C-X-C chemokine receptor type 4*
DAPI	*4',6-diamidino-2-phenylindole*
DCIS	*Ductal carcinoma in situ*
DMEM	*Dulbecco's modified Eagle medium*
DTT	*Dithiothreitol*
E-cadherin	*Epithelial cadherin*
ECM	*Extracellular matrix*
EDTA	*Ethylene diamine tetraacetic acid*
EGF	*Epithelial growth factor*
EGFR	*Epidermal growth factor receptor*
EMT	*Epithelial-mesenchymal transition*
ERK	*Extracellular signal-regulated kinase*
FADD	*Fas-associated death domain*
FasL	*Fas ligand*
FBS	*Fetal bovine serum*
FDA	*Food and drug administration*
G1 phase	*Gap 1 phase*
GAPDH	*Glyceraldehyde 3-phosphate dehydrogenase*
GDF	*Growth and differentiation factor*
GPI	*Glycosyl-phosphatidylinositol*
GRK2	*G protein-coupled receptor kinase 2*
GTP	*Guanosine triphosphate*
hTER	*Telomerase RNA template*

hTERT	*Human telomerase reverse transcriptase*
ID	*Inhibitor of DNA binding*
IL	*Interleukin*
JAK	*Janus kinase*
JNK	*c-Jun N-terminal kinase*
LAP	*Latency associated peptide*
LCIS	*Lobular carcinoma in situ*
LIF	*Leukemia inhibitory factor*
LNA	*Locked nucleic acid*
LTBP	*Latent TGFβ-binding protein*
M phase	*Mitosis phase*
M-MLV	*Moloney murine leukemia virus*
MAPK	*Mitogen activated protein kinase*
MIA	*Melanoma inhibitory activity*
miRNA	*MicroRNA*
MIS	*Mullerian inhibitory substance*
MISC	*Myeloid immune suppressor cell*
MMP	*Matrix metalloproteinase*
mTOR	*Mammalian target of rapacamycin*
MTT	*Methylthiazolyldiphenyl-tetrazolium bromide*
N-cadherin	*Neural cadherin*
NF-κB	*Nuclear factor κB*
OncomiR	*Oncogenic miRNA*
PAI	*Plasminogen activator inhibitor*
PBS	*Phosphate buffer saline*
PDGF	*Platelet-derived growth factor*
PI3K	*Phosphoinositide 3- kinase*
PNA	*Peptide nucleic acid*
PR	*Progesterone receptor*
PTEN	*Phosphatase and tensin homolog*
PTHrP	*Parathyroid hormone related protein*
RANKL	*Receptor activator of nuclear factor kappa-B ligand*
Rb	*Retinoblastoma protein*
RISC	*RNA-induced silencing complex*
RT-PCR	*Reverse transcription polymerase chain reaction*
RUNX2	*Runt-related transcription factor 2*
S phase	*Synthesis phase*
SAPK	*Stress-activated protein kinase*
SBE	*Smad binding element*
SGF	*Sarcoma growth factor*

SHIP	*Src homology 2 domain-containing 5' inositol phosphatase*
siRNA	*Small interfering RNA*
STAT	*Signal transducer and activator of transcription*
TGFβ	*Transforming growth factor β*
TIEG1	*TGFβ-inducible early-response gene*
TIMP	*Tissue inhibitor of metalloproteinase*
TN	*Triple negative*
TNF-α	*Tumor necrosis factor α*
TβRI	*TGFβ receptor type I*
TβRII	*TGFβ receptor type II*
UTR	*Untranslated region*
uPA	*Urokinase-type plasminogen activator*
uPAR	*Urokinase plasminogen activator receptor*
VEGF	*Vascular endothelial growth factor*

Abstract

Breast cancer is the most common cancer in women worldwide. The incidence of breast cancer has increased over the past decades but the mortality has decreased due to the advent of potent therapies. There are different subtypes of breast cancers that respond differently to treatment. One breast cancer subtype called triple-negative lacks three different molecular markers and is of particular interest as there are currently no effective treatments for it. The Transforming Growth Factor β (TGFβ) superfamily of growth factors comprises a large set of pluripotent cytokines involved in numerous biological processes ranging from embryonic development, growth, cell fate, differentiation and homeostasis. TGFβ signaling takes place in all cells throughout the body and it of critical importance in cancer where it can play a dual role. Indeed, in normal cells and in early stage cancers TGFβ retains its ability to maintain tissue homeostasis and acts therefor as a tumor suppressor. After further mutations affect TGFβ signaling, some of the responses are lost and TGFβ favors progression to metastasis. As such TGFβ is of major importance in breast cancer therapy as it drives cancer progression to lethal metastatic stages.

As explained in the introductory chapter of this present work, numerous approaches have been attempted to block TGFβ signaling. However the different methods have failed as they led to non-negligible side effects. After the discovery of a novel class of small non-coding RNAs called microRNA and their deregulation in cancer cells, hope for a new "miRNA correction therapy" arose. Early works indicated that modulating miRNA levels in vivo could alter cellular behavior. This thesis focuses on elucidating the role of TGFβ-regulated miRNA in breast cancer progression. This work illustrates the use of 2'O-methyl-modified oligonucleotide sense or antisense sequences to mimic or inhibit TGFβ-regulated miRNA in order to interfere with downstream TGFβ effector miRNAs thereby preventing TGFβ deleterious effects. Metastatic breast cancers typically are not always suitable for surgery typically have moderate responses to currently available chemotherapy treatments. As such, metastatic triple-negative breast cancers are not treatable and have a high mortality rates.

In the second and third chapter of this work, using a triple-negative breast cancer model in which TGFβ induced miR-181 overexpression, I showed that inhibiting this downstream effector of TGFβ using an antisense oligonucleotide decreased TGFβ-mediated pro-metastatic effects *in vitro* as measured by both cell migration and invasion without altering the beneficial tumor suppressive effects of TGFβ. In another triple-negative breast cancer model in, which TGFβ induced a down-regulation of miR-30, I showed that overexpression of miR-30 activity using sense oligonucleotides interfered with TGFβ signaling once again. These finding indicate that miRNA correction therapy can be a promising new therapeutic strategy to treat a currently untreatable subtype of breast cancer. More importantly, miRNA correction therapy can be used in other cancers. A broader screen revealed that a large subset of miRNA were regulated by TGFβ in triple-negative breast cancer cells and were potentially also targetable downstream effectors of TGFβ in breast cancer progression as shown by some preliminary experiments detailed here. In the fourth chapter of this work, I performed a broad screen of the miRNome and identified a subset of 50 TGFβ-regulated miRNA and started to characterize them functionally. This work will be the foundation of many upcoming projects downstream of TGFβ signaling.

Together, the results of this present study indicate that TGFβ acts as a potent mediator of breast cancer progression and miRNA modulation using exogenic 2'O-methyl-modified RNA can effectively be used *in vitro* to slow this progression. I have showed in two instances that targeting miRNA was an efficient method to interfere with TGFβ signaling. This research provides new insights into the development of a new therapeutic strategy for cancers. This work is intended to show the potential of miRNA-based technologies to modulate cell behavior at a broader transcriptome-level unlike current pharmaceutical strategies that aim at targeting individual pathological pathways. In the last decade, cancer cells have been shown to be highly dependent on oncogenes, I believe cancer cells are also highly dependent on oncomiRs such as miR-181. Undoubtedly, the interest of the pharmaceutical industry in miRNA-based therapies will be growing and gaining momentum. As our understanding of miRNA target transcript selection and regulation improves, synthetic miRNA will perhaps be generated to target selected oncogenic

pathways. Such crucial master regulators of the transcriptome will be major actors in the next generations of cancer therapies.

Résumé

Le cancer du sein est le cancer féminin le plus répandu au monde. L'incidence du cancer du sein a augmenté au cours des dernières décennies mais la mortalité a diminué grace aux nouvelles thérapies. Il existe différents sous-types de cancer du sein répondant différemment aux traitements. Un sous-type de cancer du sein ne disposant pas de trois marqueurs moléculaires, appelé triple-négatif, est d'un intérêt particulier puisqu'il n'existe actuellement aucun traitement efficace pouvant le cibler. La superfamille du facteur de croissance "transforming growth factor β" (TGFβ) comprend un ensemble de cytokines pluripotentes impliquées dans de nombreux processus biologiques allant du dévelopement embryonnaire à la croissance, la différentiation, à l'homéostasie. La voie de signalisation du TGFβ a lieu dans toutes les cellules du corps et a un rôle majeur dans le cancer où elle joue un rôle double. En effet, dans les cellules normales et dans les cancers de stade précoce, le TGFβ conserve son aptitude à maintenir l'homéostasie et agit ainsi tel un suppresseur de tumeur. Ensuite d'autres mutations affectant la voie de signalisation du TGFβ, certaines de ses réponses biologiques sont perdues et le TGFβ favorise alors la progression tumorale et les métastases. La cytokine TGFβ joue alors un rôle majeur dans la thérapie du cancer du sein puisqu'elle promeut la progression tumorale vers les stades métastatiques.

Le chapitre d'introduction de ce présent travail explique les nombreuses approches qui ont été tentées pour bloquer la voie de signalisation du TGFβ. Toutesfois, ces différentes méthodes ont échoué parce qu'elles donnaient lieu a des effets secondaires non négligeables. Après la découverte d'une nouvelle classe de petits ARN non codants, appelés microARN, et leur dérégulation dans le cancer, l'espoir d'une nouvelle thérapie à base de microARN est née. Des études initiales ont revelé *in vivo* que la modification des niveaux d'expression des microARN endogènes modifiait le comportement cellulaire. Ce travail de doctorat a pour but d'élucider le rôle des microARN regulés par le TGFβ dans la progression du cancer du sein.

Ce travail illustre l'utilisation d'oligonucléotides modifiés pour augmenter ou diminuer les niveaux de microARN régulés par le TGFβ afin d'interférer avec les effets délétères du TGFβ. Les cancers du sein métastatiques ne sont pas souvent opérables et peu répondants aux traitements systémiques. Les cancers métastatiques triple-négatifs entrainent une forte mortalité.

Dans le deuxième et troisième chapitre de ce mémoire, en utilisant une lignée triple-négative du cancer du sein dans laquelle le TGFβ induisait le miR-181, j'ai demontré que l'inhibition de ce miR-181, à l'aide d'oligonucléotides antisens, diminuait les effets prométastatiques du TGFβ *in vitro* démontré par une diminution du potentiel promigratoire et proinvasif du TGFβ. Ceci n'affectait pas pour autant les effets suppresseur de tumeur du TGFβ. Dans une autre lignée triple-négative dans laquelle le TGFβ diminuait l'expression du miR-30, j'ai demontré que la surexpression du miR-30 à l'aide de séquences sens oligonucléotidiques perturbait la voie de signalisation du TGFβ à nouveau. Ces résultats indiquent que la thérapie à base de microARN est une nouvelle stratégie thérapeutique prometteuse pour les sous-types actuellement intraitables. Une étude plus vaste révele qu'une grande proportion des miARN est regulée par le TGFβ dans les cancer du sein triple-négatif. Ces miARN sont potentiellement des effecteurs du TGFβ et constituent donc des cibles d'intérêt thérapeutique comme le suggère les résultats préliminaires détaillés dans ce mémoire. Dans le quatrième chapitre de ce travail, j'ai testé l'effet du TGFβ sur le microRNome, identifié une cinquantaine de miARN régulés par la cytokine et ai commencé à les caractériser fonctionnellement. Ce travail servira de base pour de nombreux projets à venir sur la voie de signalisation du TGFβ.

Prise dans son integralité, cette recherche doctoratale démontre que le TGFβ est moteur de la progression tumorale du cancer du sein et que l'utilisastion d'ARN exogènes modifiés en 2'O-méthyl est efficace *in vitro* pour ralentir la progression cancéreuse. J'ai montré à deux reprises que le fait de cibler les miARN était une méthode efficace pour interférer avec une des voies de signalisation du TGFβ sans nécessairement affecter les autres. Cette étude contribute au développement de nouvelles stratégies thérapeutiques centrées sur les miARN permettant d'affecter les cellules cibles à l'échelle du transcriptome. Au cours de la derniere décennie, il a été montré que les cellules

cancéreuses étaient souvent dépendantes d'oncogènes. Je pense qu'elles le sont également d'oncomiARN tells que le miR-181. Les industries pharmaceutiques montre un intérêt grandissant pour les thérapies à base de miARN. Alors que notre compréhension des relations entre miARN et leurs messagers cibles s'affine, il sera possible de créer des miARN synthétiques capables de cibler les voies oncogéniques particulières. De tels régulateurs majeurs du transcriptome vont être des acteurs clefs des nouvelles générations de thérapies du cancer.

Contribution of authors

I wrote the present thesis and performed the experiments that produced the results in each of these manuscripts under the supervision of my research supervisor Dr. Jean-Jacques Lebrun.

Acknowledgements

These 7 years have been a challenging experience and have helped me learn invaluable lessons both professionally and privately. This is the beginning of a great life of research.

I would like to thank my supervisor, Dr. Jean-Jacques Lebrun providing me with the opportunity to learn new technical skills in his laboratory and become a more mature research scientist. I would like to thank my PhD advisory committee members for their: Dr. Bertrand Jean-Claude, Dr. John di Battista, Dr. Suhad Ali and my supervisor. I also wish to thank Dr. Sabah Hussain and his graduate student Raquel Echavarria for the intense discussions we had together which led to a co-authored paper.

I thank Laure Humbert who was an outstanding professional collaborator and source of moral support during these last years. I enjoyed agreeing and disagreeing with her during our countless fruitful scientific discussions. I also thank Jay Bhat and Roberta Carin for managing some key logistical aspects of the PhD life over the years. The staff from the Royal Victoria Hospital receiving department was always very pleasant and constantly reminded me about the world outside the research facility.

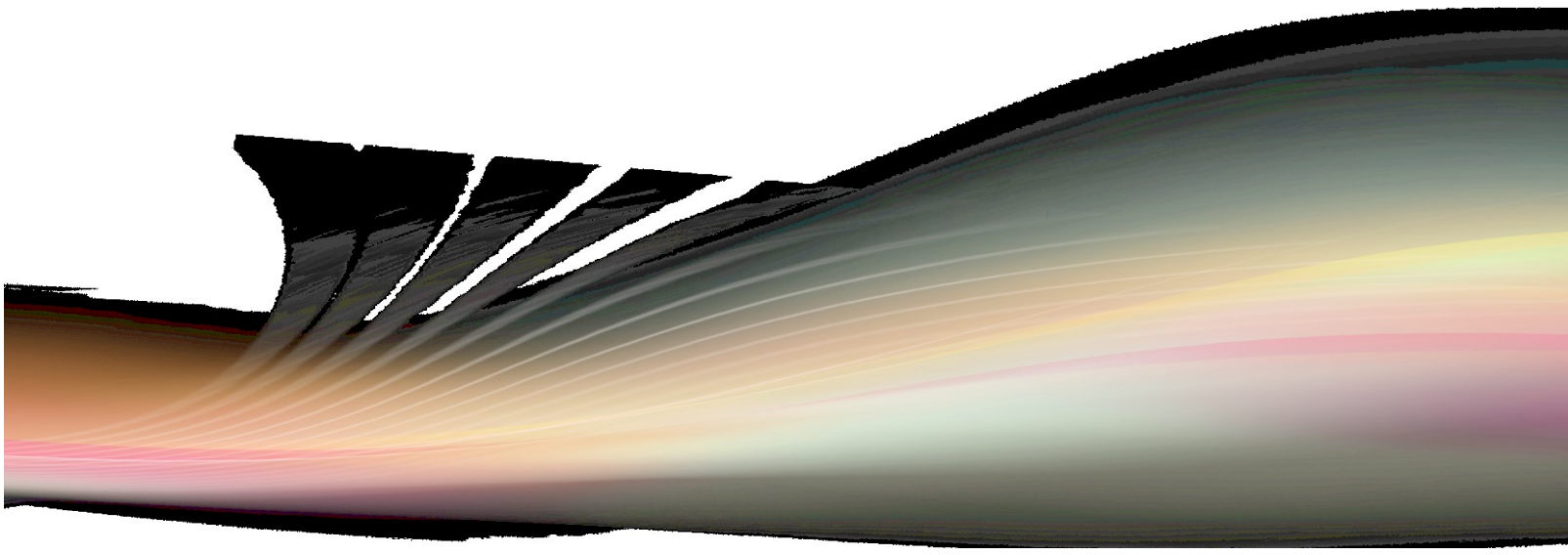
I want to thank the division of Experimental Medicine and specifically Marylin Linhares and Dominique Besso for their initial help when entering this division of the Faculty of Medicine.

I also want to thank my parents Bertrand and Annie for the education and respect for hard work that they bequeathed to me which allowed me to reach this point. I obviously wouldn't be here without them. I want to thank my brother Julien and his wife Marie for giving me courage when I needed it.

I want to thank my Persian princess Zohreh for patiently putting my pieces back together when I was shattered. Thank you for all the support. You are my future.

Chapter 1

Introduction



1.1.Breast carcinoma: A complex disease

Breast adenocarcinoma is a cancer that most often arises from the inner lining of the duct or the tubule, which provides milk to the milk duct. These cancers are respectively called ductal or lobular carcinoma.

1.1.1. Milk ducts and lobules and their role in the breast

1.1.1.1.The mammary duct: a structure containing different cell types

Breasts are hormone-sensitive organs; they experience structural changes during the reproductive cycle. The breast acts as an apocrine gland that initially provides essential immunity in colostrum or first-milk to the infant and as such is a major element in offspring nurturing.

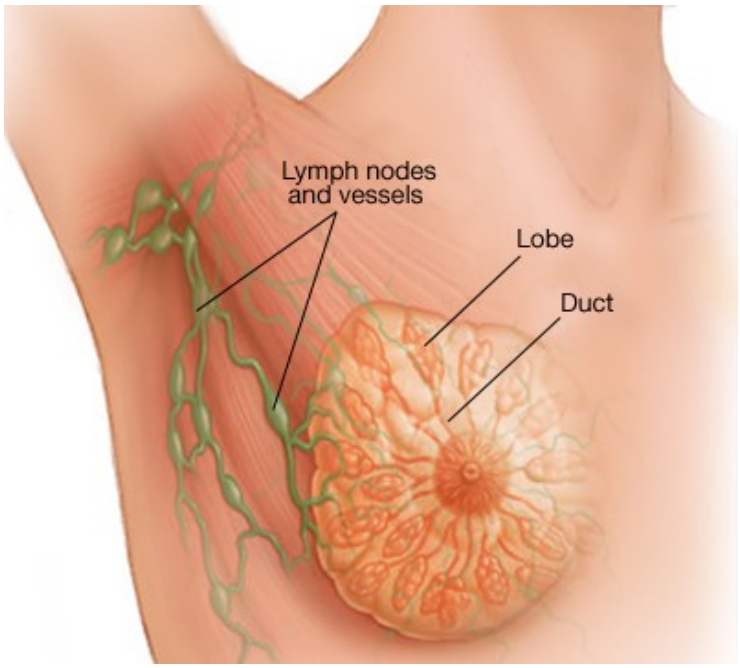
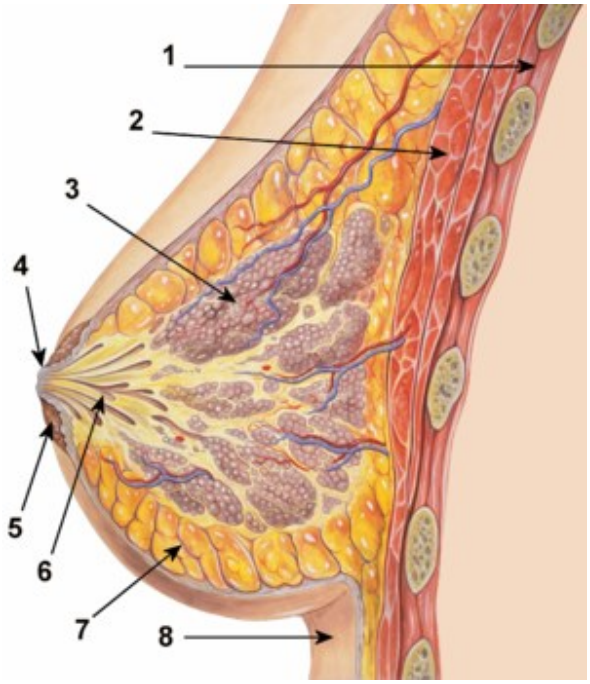


Fig. 1.1. Schematic representation of normal breast

(From mayoclinic.com)

Approximately 75% of the lymph from the breast travels to the axillary lymph nodes (Fig 1.1) which include the pectoral, subscapular, and humeral lymph-node groups.



(From http://www.infraredmedicalsolutions.com)

Fig. 1.2. Schematic representation of normal breast

1. Chest wall; 2.Pectoralis muscle; 3.Lobules; 4.Nipple; 5.Areola; 6.Milk duct; 7. Fatty tissue; 8.Skin

The nutrients are provided by the blood to the lobules and the epithelial cells lining the duct synthetize caseins secreted in the milk duct which reach the nipple where the infant feeds from (Fig.1.2).

1.1.1.2.Lactation: milk production and secretion

Upon nipple suction, released oxytocin leads to milk flow from the alveoli, through the milk ducts into the milk sacs behind the areola. Human breast development is dependent on the presence of ovarian steroids. The secretory alveoli subunits continue to respond to steroids throughout the reproductive years. Lactogenesis is triggered by a rapid and drastic fall in progesterone at delivery and maintained by prolactin (PR) while the actual expulsion of milk depends on oxytocin. Upon suction, the afferent neuron signals the neurosecretory cells in the hypothalamus to secrete hormones including oxytocin from the posterior pituitary into the blood. This short-term response is followed by a long-term response, which is initiated immediately after birth; the hypothalamus detects the drop in progesterone releases PR into the blood thereby maintaining the breast in a lactating state.

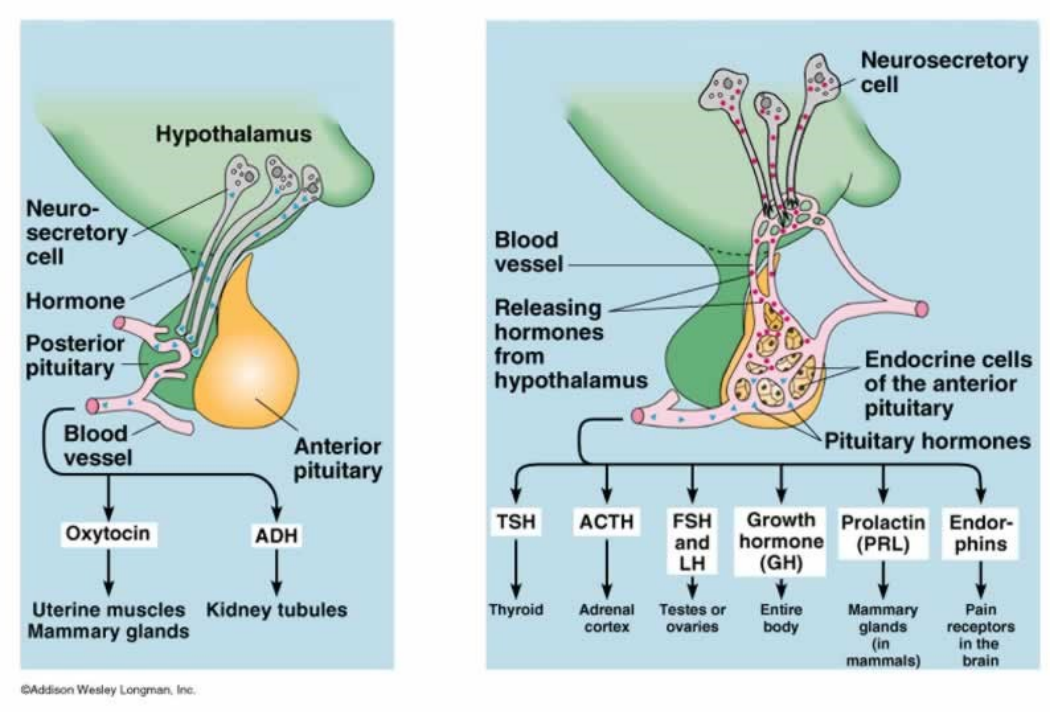


Fig. 1.3. Lactation physiology

(From Martini, Fredric, H. 2006. Fundamentals of anatomy and physiology. 7th ed. Pearson Education Inc. USA)

Nipple suction triggers hypothalamic oxytocin release (Fig.1.3) and maintains the pituitary gland PR production.

1.1.2. Breast carcinoma: from the origin to treatments

1.1.2.1.Epidemiology

Breast cancer (BC) incidence rate in women in Canada rose steadily from the early 1980s to the late 1990s due to increased mammography screening. Also increasing use of hormone replacement therapy among post-menopausal women has been linked to a higher risk of BC (www.cancer.ca). BC death rates in women have decreased across all age group since the mid-1980s due to screening and therapeutic improvements. Ductal carcinoma is the most common type of BC. In 2012, 22,700 women were diagnosed with BC. This represented 26% of all new cancer cases of, which 5,100 women died from BC, which represented 14% of all cancer deaths in women. On average, 62 Canadian women

will be diagnosed with BC every dayand 14 will die. While for mean, 200 will be diagnosed annually with BC and 55 will die from it (Table 1.1).

Category	Males	Females
New cases	200	22,700
Incidence rate (for every 100,000 people)	1	96
Deaths	55	5,100
Death rate (for every 100,000 people)	<0.5	19
5-year relative survival (estimates for 2004–2006)	79%	88%

Table. 1.1. Canadian breast cancer statistics

(www.cancer.ca)

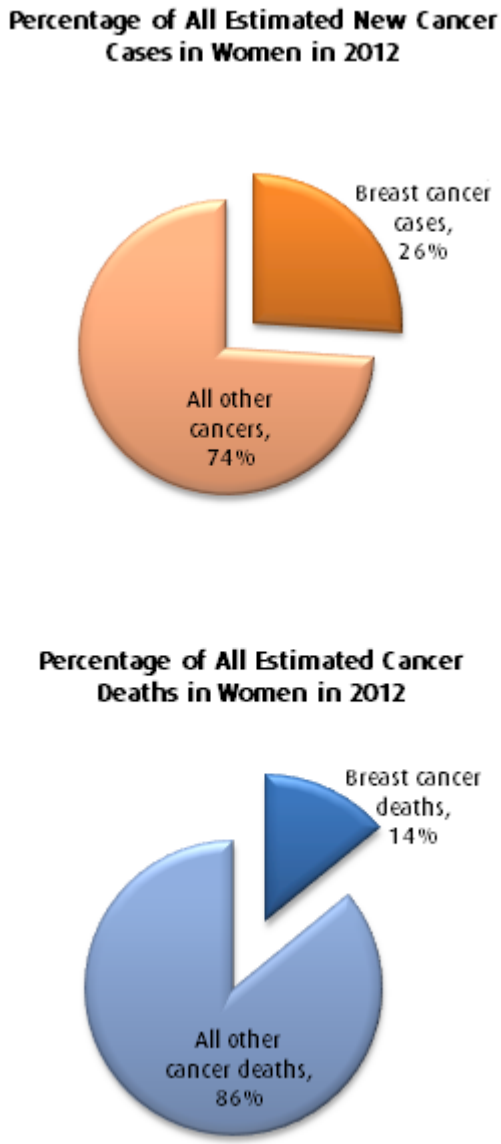


Fig. 1.4. New breast cancer cases and deaths in Canada in 2012

(www.cancer.ca)

BC represents 26% (Fig.1.4) of all new cancer cases in 2012. About 1 in 9 Canadian women is expected to develop BC during her lifetime and 1 in 29 will die from it.

1.1.2.2.Etiology

Most BC occur in women because of the sensitivity of this tissue to estrogen and progesterone. Estrogen is associated with BC and encourages the cancer growth. Interestingly, as far as location is concerned BC is more common in high income, developed countries such as Canada, the United States and some European countries like Great Britain. The risk of developing BC increases with age. Age of onset for BC in women is between the ages of 50 and 69.

Risk factors include BC family history, dense breast, BRCA gene mutations, early menarche, late/no pregnancies, late menopause, use of hormone contraceptives or replacement therapy, alcohol and obesity.

1.1.2.3.Development of malignant breast cancer

Transformations can either be benign (ex: non-invasive intraductal papilloma) or malignant (ex: invasive ductal carcinoma).

The most common type of malignant BC is adenocarcinoma occurring in the upper, outer part of the breast, typically ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS) respectively.

Triple negative breast cancer (TN) gets its name from the fact that the cancer cells test negative for 3 markers: estrogen receptors (ER), progesterone receptors (PR) and HER2 overexpression. Standard treatments for BC, such as hormonal therapy or trastuzumab (Herceptin) cannot be used with this type of BC. Basal-like (BL) BC have a certain genetic pattern. BL cells overexpress cytokeratin5/6 and epidermal growth factor receptors (EGFRs). BL cells are often TN. To date, there is no accepted definition for BL cancers, and no clinically available genetic test to identify such tumors. TN tumors are most often invasive ductal cancers with worst prognosis (Fig.1.5) and higher early recurrence rates (Fig.1.6), but ductal carcinoma *in situ* may also be TN. TN and BL cancers also differ from other BC types. Most are high-grade and aggressive tumors diagnosed at a later stage when metastasis has already occurred.

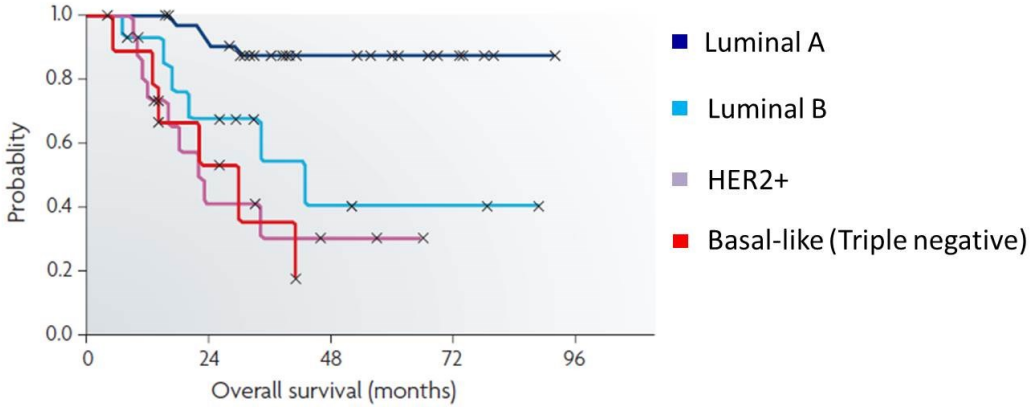


Fig. 1.5. Overall survival depending on breast cancer type

(From Vargo-Gogola, Nature Rev. Cancer 2007)

These tumors metastasize to the brain or lung more frequently than other types of BC, which target bones or liver. TN and BL tumors respond to chemotherapy at first, but typically recur within 5 years of treatment. The prognosis for TN and BL tumors is less favorable, which is why they are the main focus of this thesis. TN breast cancers appear to also respond to ixabepilone. This is an exciting result because these types of tumors generally have limited treatment options[1-4]. As triple-negative breast cancer tumors have no available treatment, we investigated the possibility that cancer-deregulated miRNA could provide new therapeutic avenues.

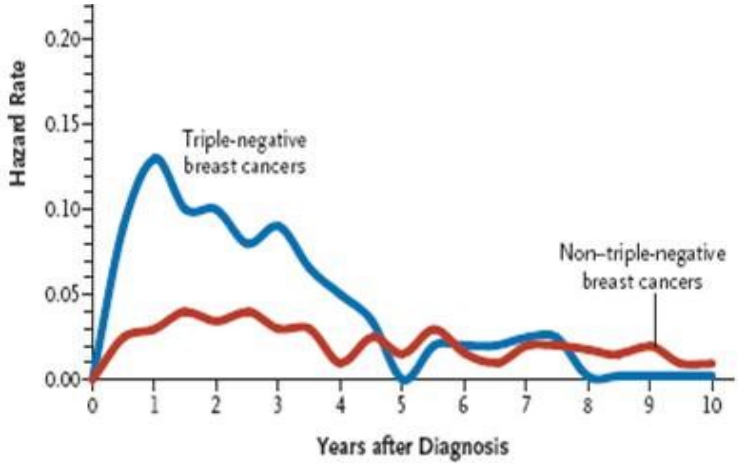


Fig. 1.6. Recurrence over time (TNBC vs. others)

(From Foulkes, N. Engl. J. Med. 2010)

1.1.2.4.Diagnosis, prognosis, treatment

Diagnosis is given after using different complementary methods to conclude the presence of cancer after ruling out other causes for the symptoms. A **physical examination** allows the doctor to look for any signs of BC. The doctor checks for breast lumps, hardening or thickening in the tissue, skin changes, including dimpling, changes in the nipples and lymph nodes for signs of swollen axillary lymph nodes.

This is followed by a x-ray **diagnostic mammography** to follow up on abnormal results of a clinical breast examination, diagnose BC and help find an abnormal area to be sampled during a biopsy**. Blood chemistry tests** detect abnormalities and stage breast cancer. Alanine aminotransferase, aspartate transaminase and alkaline phosphatase reflect liver function. Alkaline phosphatase indicates bone metastatis. A **biopsy** is sometimes conducted to determine molecule and histological features of the abnormal tissue. Tissues or cells are isolated for a pathology report and will confirm whether or not cancer cells are present in the sample. A breast biopsy is the only definite way to diagnose BC. Most biopsy samples taken from breast lumps are found to be benign (non-cancerous). A **lymph node biopsy** removes lymph nodes during a surgical procedure so they can be examined under a microscope to find out if they contain cancer. With BC, axillary lymph nodes are removed. BC cells can travel through the lymph system, and the first place they may spread is to these lymph nodes. The number of positive lymph nodes helps to determine the stage of BC. A **bone scan** using bone-seeking radioactive materials provides a picture of the bones and tests for bone metastases. A bone scan may be recommended if blood alkaline phosphatase levels are highIf the blood chemistry tests show increased levels of liver enzymes, a **liver ultrasound** may be done to test for liver metastases.

The biopsy also allows for **molecular marker testing**. Hormone receptor testing determines the amount of estrogen receptor and progesterone receptor in the BC cells.

Prognostic factors

The **stage of BC** is an important prognostic factor. Lower stages have less risk of the cancer recurrence and a more favorable prognosis. The most important prognostic factor for BC is **axillary lymph nodes status**. The greater the number of positive lymph nodes, the higher the risk of recurrence is. The **size of a breast tumor** is the second most important prognostic factor for BC. The tumor size is an independent prognostic factor, regardless of the lymph node status. The size of the tumor increases the risk of recurrence. Large breast tumors (≥5 cm) have the greatest risk of recurrence. Breast tumors ≤1 cm with no positive lymph nodes have the most favorable prognosis. Low-grade tumors often grow slower and are less likely to spread. **Hormone receptor status** is a predictor of hormonal therapy response. Estrogen receptor–positive (ER+) and progesterone receptor–positive (PR+) tumors are often less aggressive, low-grade tumors less likely to spread than hormone receptor negative (HR–) tumors. HER2 status is another prognostic factor and may also be used to predict therapeutic responses. HER2-overexpressing tumors tend to be higher grade tumors more likely to spread. Women with HER2-positive tumors may not respond well to hormonal therapies like tamoxifen (Novadex, Tamofen) but are likely to respond well to a drug called trastuzumab (Herceptin). A woman's **age** at the time of her breast cancer diagnosis can affect the prognosis. Younger women usually have a greater risk of recurrence and a poorer overall prognosis. Younger women with BC tend to have more aggressive, higher-grade cancer and more advanced BC at the time of diagnosis. **Gene expression status** can be determined using FDA-approved MammaPrint. Studies have shown that mRNA profiling could predict tumor sensitivity to chemotherapy[5,6] or endocrine therapy[7,8].

Treatment

Treatment decisions are based on the stage of the BC if the woman has reached menopause, the hormone receptor status, the HER2 status, the risk for recurrence, the overall health, and the woman's personal decision about certain treatments.

Treatment options include surgery, radiation therapy, chemotherapy, hormonal therapy, biological therapy. Treatments vary according to cancer stage.

Stage 0: Ductal carcinoma *in situ* (DCIS) is the most common type of non-invasive BC. Nearly all women diagnosed with this early stage BC can be successfully treated. Breast-conserving surgery is the primary treatment for DCIS and lymph nodes are not usually removed. Hormonal therapy may be offered after surgery for women with DCIS who have hormone receptor–positive BC and are at a high risk of the BC recurring. Women with DCIS are not offered chemotherapy. *Stage I and II*: Surgery is the primary treatment. The risk of BC recurrence will influence the type of adjuvant therapy offered. External beam radiation therapy is almost always offered after surgery. It may also be offered after a mastectomy if the tumor has invaded the skin or muscles of the chest wall. If given, chemotherapy is independent hormone receptor status. Hormonal therapy may be offered to women who have Stage I or II BC that is hormone receptor positive (ER+, PR+ or both). Tamoxifen (Nolvadex, Tamofen) is the most common anti-estrogen drug and aromatase inhibitors letrozole (Femara), anastrozole (Arimidex) and exemestane (Aromasin) are used only in post-menopausal women. *Stage III*: The types of treatments given are based on the unique needs of the woman with cancer. At this advanced stage, the tumor can sometime not be operable if the tumor is attached to the chest wall or skin, the lymph nodes are attached to other structures in the armpit or the cancer has spread to a supraclavicular lymph node. Chemotherapy is the primary treatment for Stage III BC and may be offered after BC surgery or before surgery to shrink the tumor. Stage IV: At present, Stage IV (or metastatic) BC is not considered curable. The goal of treatment is to help the woman survive and to remain free of symptoms as long as possible. The type of treatment offered for Stage IV BC will depend on the hormone receptor status of the tumor, the HER2 status of the tumor. The response of Stage IV BC to single chemotherapy drugs or combinations is similar. Single drugs are often given for Stage IV BC because they have fewer side effects than combination chemotherapy. Bisphosphonates such as clodronate (Bonefos), pamidronate (Aredia) or zoledronic acid (Zometa, Aclasta) may be used to reduce pain and fractures when BC has spread to the bones. When given every 6 months, denosumab increases bone density in women[9,10].

1.2.Transforming growth factor β: an ubiquitous cytokine with complex functions

The transforming growth factor-beta (TGFβ), discovered nearly three decades ago, was isolated as a secreted protein from cells infected with the sarcoma virus[11-13]. TGFβ was initially shown to give normal fibroblasts transient phenotypic properties of transformed cells, such as anchorage-independent growth in soft agar[11].

1.2.1. TGFβ ligands

More than 40 different TGFβ family members (Table1.2) have been identified, including the activin/inhibin subfamily, the bone morphogenetic proteins (BMPs), nodal, myostatin, and the mullerian inhibitory substance (MIS)[14-17]. As for the TGFβ subfamily, three distinct isoforms have been identified (TGFβ-1, -2, -3), each encoded by a different gene[17-20], 8–10]. The three proteins share large regions of sequence similarity making them 70% homologous. Although all isoforms are expressed in all tissues, most studies have been conducted on TGFβ-1. The active molecule is a homodimer stabilized by hydrophobic interactions strengthened by a disulfide bond. The active form of the TGFβ molecule is a homodimer stabilized by hydrophobic interactions and interlocked by a disulfide bond which forms the cysteine knot[21]. TGFβ is synthesized as the C-terminal domain of a precursor form that is cleaved prior to secretion. This precursor is an inactive latent complex consisting of a TGFβ dimer in a non-covalent complex with two segments linked to latent TGFβ-binding proteins[22]. One of these binding proteins is the TGFβ pro-peptide or latency associated peptide (LAP) that remains bound to TGFβ after secretion, retaining TGFβ in an inactive form. The second binding protein is latent TGFβ-binding protein (LTBP), which is linked to LAP by a disulfide bond. The TGFβ precursor complex is released in the extracellular matrix that acts as a reservoir. The inactive form of TGFβ can be activated by multiple processes. The maturation of TGFβ can indeed result from the enzymatic activity of proteases (furins, plasmin, calpain, etc.), physical and chemical treatments (acidification, heat, reactive oxygen species, etc.), as well as binding to mannose-6-phosphate receptor. TGFβ activation is also regulated by glycosidases, thrombospondin and some therapeutic molecules (anti-estrogens, retinoic acids, etc.)[22-24].

1.2.2. TGFβ signal transduction

1.2.2.1.The Smad-dependent pathway

Three decades ago, Massagué *et al.* identified high-affinity cell surface receptor (type I receptor) for TGFβ[25]. Using an affinity cross-linking approach, other TGFβ type II and type III receptors were identified[26]. TGFβ was later shown to control and modulate a plethora of biological effects, ranging from cell growth and differentiation, embryogenesis, immunity, reproduction, hormonal synthesis and secretion, bone formation, tissue remodeling and repair, and erythropoiesis, among others[14,16,20,27,28]. TGFβ signal transduction is initiated with ligand binding to a transmembrane constitutively auto-phosphorylated serine/threonine receptor kinase termed type II receptor (TβRII) (Fig. 1.7)[20]. Upon ligand binding, the chain of conformation of the type II receptor leads to the recruitment and transphosphorylation of the type I receptor (TβRI) within its juxtamembrane glycine and serine-rich region, thereby activating the kinase activity of the TβRI[15,16]. The activated TβRI then phosphorylates intracellular mediators receptor-regulated Smads (R-Smads), Smad2 and Smad3, at their C-terminal serine residues. This phosphorylation event allows for subsequent heterotrimerization of two phosphorylated R-Smad with one common partner, Smad4[29-32]. Smads are homologues of Drosophila gene *Mad* (mothers against decapentaplegic) and of *sma* genes in *C. elegans*. Activin and TGFβ share the same R-Smad signaling molecules, but other members of the TGFβ superfamily signal through distinct R-Smad proteins following activation of their cognate receptors such as Smad1, 5 and 8 for BMP ligands (Table 1.2)[17]. Smad heterotrimers later translocates to the nucleus where they interact with DNA with poor affinity[33]. In order to increase the binding to the DNA, associated proteins can act as chaperones and facilitate the process[34]. Nuclear import of Smads is regulated by importin-dependent and importin-independent mechanisms[35,36]. DNA binding motif for Smads called Smad-binding element (SBE) is CAGAC. The partner proteins, which act as co-activators or co-repressors have a differential expression pattern in space and in time, thereby providing tissue and cell type specificity to TGFβ[37,38].

1.2.2.2.The Smad-independent pathways

The Smad pathway is the canonical signaling pathway for TGFβ ligands, but other TGFβ-activated intracellular signaling cascades have been identified (**Fig. 1.8**). The stress-activated kinases p38 and JNK (Jun N-terminal Kinase) is induced by TGFβ ligands and interacts with Smad signaling leading to apoptosis and epithelial-mesenchymal transition (EMT)[39-44]. The p38 kinase pathway also plays an important role in the signaling of activin and was shown mediate part of activin-mediated cell growth arrest in breast cancer[42] and activin-mediated Pit-1 down regulation in pituitary tumors[44]. TGFβ signaling is also carried out through the mitogen activated protein kinase (MAPK) pathway with the activation of extracellular-signal-regulated kinases 1 and 2 (ERK1 and ERK2), which induces EMT[40,45,46]. Rho GTPases have been shown to relay the TGFβ signals which cause the necessary cytoskeleton reorganization for cell motility, and invasion, through activation of RhoA, Cdc42, and Rac[47,48]. TGFβ also signals through the mTOR and the phosphoinositide 3-kinase (PI3 K)/Akt pathway to regulate cell growth inhibition[49] and EMT[50,51]. The Smad pathway is the core of TGFβ signaling, however studies have indicated that other non Smad pathways mediate signaling downstream of these receptors (Fig. 1.10)[52] The mitogen activated protein kinase (MAPK) pathway leads to the phosphorylation of ERK1 and 2 through Src, Raf, and MEK, which leads to epithelial-mesenchymal transition (EMT)[40,45,46] The p38/JNK (Jun N-terminal Kinase) pathway induces MKK activation through TRAF6 ubiquitination, thereby leading to EMT and apoptosis[40-44,48,53]. The Rho-GTPase pathway involving RhoA, Cdc42, and Rac affects reorganization of cell cytoskeleton essential in cell motility[47,48]. The phosphoinositide 3-kinase (PI3K)/Akt pathway inhibits cell growth[49] and promotes EMT[50,51] through mammalian target of rapamycin (mTOR). The above-mentioned Smad-independent pathways are essential in the dual role of TGFβ as they participate in the pro-metastatic effects of the cytokine.

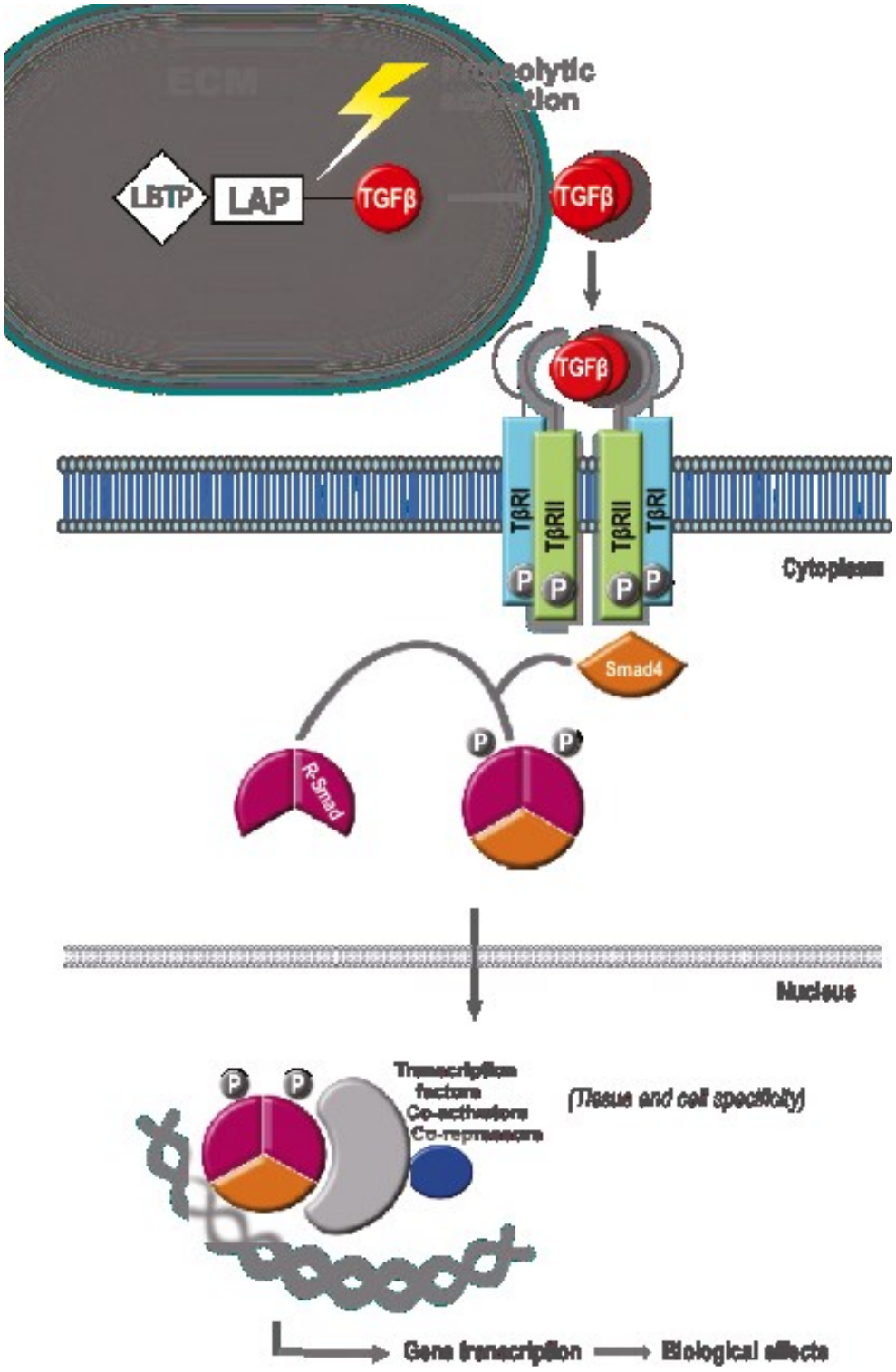


Fig. 1.7. The TGFβ/Smad canonical signaling pathway.

(Adapted from Neel *et al.*, 2012[54])

The active TGFβ ligand is a dimeric molecule composed of two monomers linked by a disulfide bridge and hydrophobic interactions. Each TGFβ subunit is synthesized as a large inactive precursor molecule bound to accessory proteins (LAP and LTBP). This precursor is stored in the extracellular matrix (ECM) and can be rapidly cleaved and activated to become bioavailable. Signal transduction starts with ligand binding to a complex of specific serine/threonine kinase receptors (type I, type II). The type II receptor is constitutively autophosphorylated and, upon ligand binding, transphosphorylates the juxtamembrane region of the type I receptor. This is followed by phosphorylation and recruitment of the R-Smads to the type I receptor and phospho-R-Smad complex formation with common partner Smad4 in the cytoplasm. The Smad complex is then translocated to the nucleus where it interacts with various transcription factors, coactivators, or corepressors to regulate target gene expression.

Ligand	Type II receptor	Type I receptor	R-Smad	Co-receptor
TGFβ-1	TβRII	ACVRLI (ALK1) ACVRI (ALK2) TβRI (ALK5)	Smad1, 5, 8 Smad1, 5, 8 Smad 2, 3	TβRIII, endoglin, cripto
TGFβ-2	TβRII	ACVRI (ALK2) TβRI (ALK5)	Smad1, 5, 8 Smad 2, 3	TβRIII
TGFβ-3	TβRII	ACVRLI (ALK1) ACVRI (ALK2) TβRI (ALK5)	Smad1, 5, 8 Smad1, 5, 8 Smad 2, 3	TβRIII, endoglin
Activin-A	ACVR2, ACVR2B	ACVRLI (ALK1) ACVRI (ALK2) ACVR1B (ALK4)	Smad1, 5, 8 Smad1, 5, 8 Smad 2, 3	endoglin
Activin-B	ACVR2, ACVR2B	ACVR1B (ALK4)	Smad 2, 3	-
Activin-C	ACVR2, ACVR2B	ACVR1B (ALK4)	Smad 2, 3	-
Myostatin	ACVR2, ACVR2B	ACVR1B (ALK4)	Smad 2, 3	-
Nodal	ACVR2B	ACVR1B (ALK4) ACVR1C (ALK7)	Smad 2, 3 Smad 2, 3	cripto
GDF-1	ACVR2	ACVR1B (ALK4)	Smad 2, 3	cripto
GDF-11	ACVR2 ACVR2B	ACVR1B (ALK4)	Smad 2, 3	
GDF-5	ACVR2, ACVR2B, BMPR2	BMPR1B (ALK6)	Smad1, 5, 8	TβRIII
GDF-6	BMPR2	BMPR1B (ALK6)	Smad1, 5, 8	-
GDF-9b	ACVR2, BMPR2	BMPR1B (ALK6)	Smad1, 5, 8	-
Inhibin A	ACVR2, ACVR2B	-	-	TβRIII
Inhibin B	ACVR2, ACVR2B	-	-	TβRIII
Lefty-1, -2	ACVR2, ACVR2B	-	-	cripto
BMP-2	ACVR2, ACVR2B, BMPR2	BMPR1A (ALK3) BMPR1B (ALK6)	Smad1, 5, 8 Smad1, 5, 8	TβRIII, endoglin, RGMA, RGMB, HJV
BMP-4	BMPR2	BMPR1A (ALK3) BMPR1B (ALK6)	Smad1, 5, 8 Smad1, 5, 8	TβRIII, RGMA, RGMB, HJV
BMP-6	ACVR2, ACVR2B, BMPR2	ACVRI (ALK2) BMPR1A (ALK3) BMPR1B (ALK6)	Smad1, 5, 8 Smad1, 5, 8 Smad1, 5, 8	-
BMP-7	ACVR2, ACVR2B, BMPR2	ACVRI (ALK2) BMPR1A (ALK3) BMPR1B (ALK6)	Smad1, 5, 8 Smad1, 5, 8 Smad1, 5, 8	TβRIII, endoglin
BMP-9	ACVR2, BMPR2	ACVRLI (ALK1)	Smad1, 5, 8	-
MIS	AMHR2	ACVRI (ALK2) BMPR1B (ALK6)	Smad1, 5, 8 Smad1, 5, 8	-

Table 1.2. TGFβ superfamily signaling molecules.

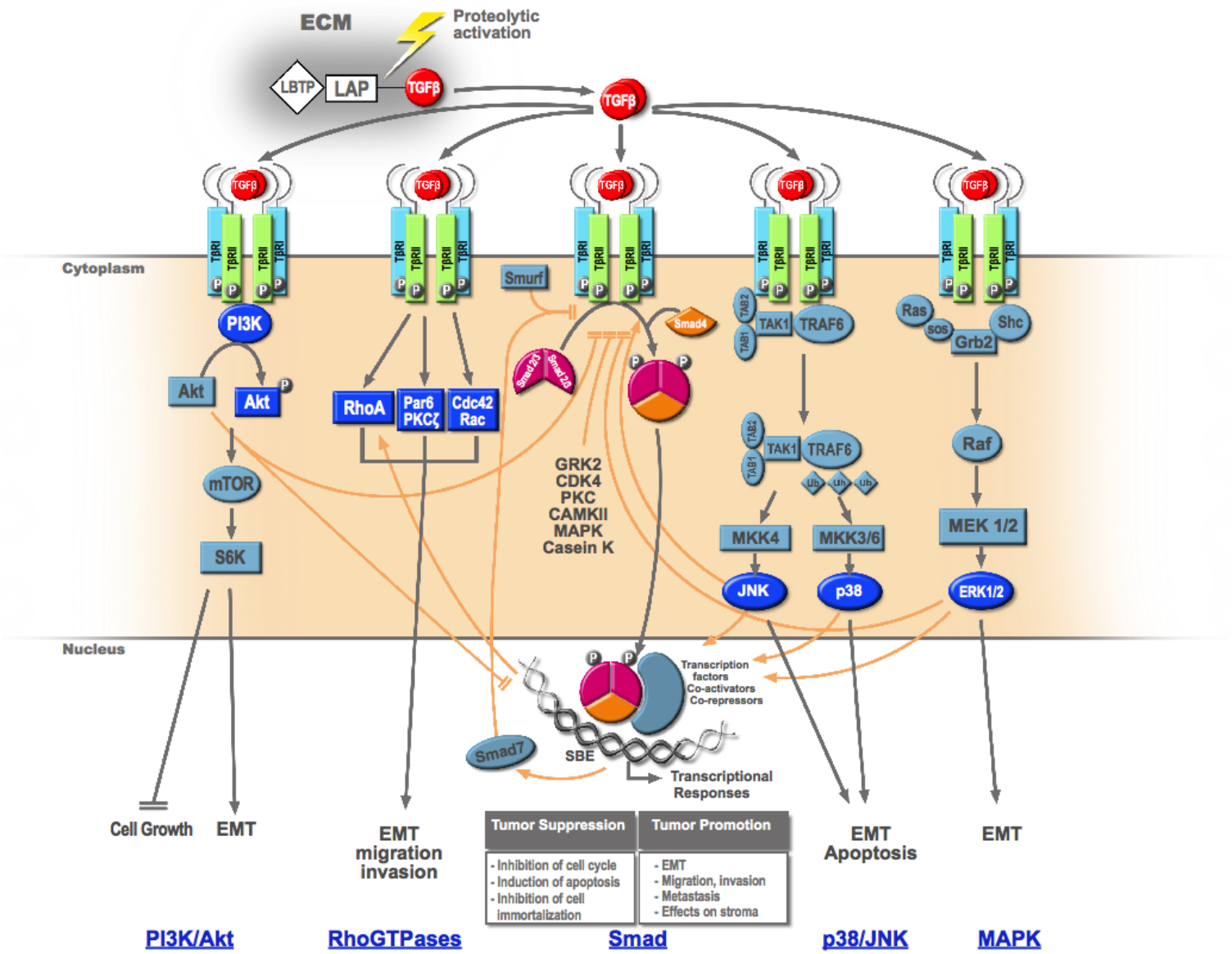
(Adapted from Humbert *et al.*, 2010[14])

TGF = Transforming Growth Factor; GDF = Growth and Differentiation Factor; BMP = Bone Morphogenic Protein; MIS = Mullerian Inhibiting Substance; RGM = Repulsive Guidance Molecule; HJV = Hemojuvelin

Fig. 1.8. The Smad-independent TGFβ signaling pathways.

(Adapted from Neel *et al.*, 2012[54])

The canonical Smad pathway is responsible for most of the TGFβ biological responses leading to tumor suppression (growth arrest, apoptosis, and prevention of immortalization) and tumor promotion (EMT, migration, invasion, and metastasis). TGFβ can activate the PI3K/Akt, RhoGTPases, MAPK, and stress-activated kinase (p38/JNK) pathways, leading to various biological effects. Depicted by the orange arrows, these pathways also cross-talk or synergize with the Smad pathway to antagonize or potentiate TGFβ signaling, respectively. Several Smad inhibitory pathways are also indicated, including TGFβ-induced gene expression of the inhibitory Smad7, and R-Smad linker phosphorylation by intracellular protein kinases (GRK2, CDK4, PKC, CamKII, MAPK, and Casein kinase).


ECM
Proteolytic activation
LBTP
LAP
TGFβ
Cytoplasm
Nucleus
PI3K
Akt
mTOR
S6K
RhoA
Par6 PKCζ
Cdc42 Rac
Smurf
Smad4
Smad2/3
GRK2
CDK4
PKC
CAMKII
MAPK
Casein K
TAB2
TAB1
TAK1
TRAF6
Ub
MKK4
MKK3/6
JNK
p38
Ras
SOS
Grb2
Shc
Raf
MEK 1/2
ERK1/2
Transcription factors
Co-activators
Co-repressors
SBE
Smad7
Transcriptional Responses
Cell Growth
EMT
EMT migration invasion
Tumor Suppression
- Inhibition of cell cycle
- Induction of apoptosis
- Inhibition of cell immortalization
Tumor Promotion
- EMT
- Migration, invasion
- Metastasis
- Effects on stroma
EMT Apoptosis
EMT
PI3K/Akt
RhoGTPases
Smad
p38/JNK
MAPK

1.2.2.3.Terminating TGFβ signalling

As most processes, TGFβ requires a negative feedback process to put an end to signaling. TGFβ induces Smad7, which sterically hinders Smad2 and Smad3 from kinase domain of TβRI[55,56]. Smad7 may also act directly as its expression correlates with tumor size both in colorectal[57] and breast cancer[58]. Smad7 also brings phosphatases and ubiquitin ligases (Smurf1/2) to the activated TGFβ receptor leading to the termination of TGFβ signaling process[59-62]. Clathrin-independent and clathrin-dependent receptor internalization mechanisms can terminate signaling[63-65], ubiquitin-mediated degradation or dephosphorylation of Smad2 and Smad3[66-69], phosphorylation of the linker domain of Smads by intracellular kinases, such as the MAPK kinases[70,71], calcium-calmodulin-dependent protein kinase II[72], cyclin-dependent kinase CDK2/4[73], casein kinase[74], protein kinase C[75], and G protein-coupled receptor kinase 2 (GRK2)[76].

1.2.3. The dual role of TGFβ in cancer

The dual role of TGFβ, also called paradox, is due to the fact that TGFβ acts both as a tumor suppressor and a tumor promoter.

1.2.3.1.TGFβ inhibits tumor progression

TGFβ inhibits growth in different cell types from epithelial, endothelial, myeloid, and lymphoid origins[17,77,78]. This defines TGFβ as an effective tumor suppressor. TGFβ acts as a tumor suppressive functions through by arresting the cell cycle, inducing apoptosis and preventing immortalization (Fig. 1.9).

Induction of cell cycle arrest

Induction of cell dependent kinase inhibitors: Cell cycle progression requires the activation of cyclin dependent kinases (CDK) by associating cyclins. As such CDK2/4/6 promote cell cycle progression[79]: their activation in G1 requires CDK4/6 association to cyclin D, and of CDK2 to cyclin A/E. At that point, the CDKs trigger the transcriptional expression of cell cycle regulators (DNA polymerase, oncogenes, etc.). Inhibitors of CDK inactivate CDK-cyclin complexes by interfering with the CDK kinase activity. Cell cycle arrest in G1 phase is induced through CDK inhibitors $p15^{INK4B}$ [80]or $p21^{KIP1}$ [81]. p15 binds

CDK4/6 or CDK-cyclin complexes[82,83]. p21 also binds CDK-cyclin complexes. p15 association with CDKs leads to loss of activity and additionally displaces p21 or $p27^{KIP1}$ thereby allowing these proteins to bind CDK-cyclin[84]. TGFβ induces gene transcription of $p15^{INK4B}$ and $p21^{KIP1}$ through Smadsand the Sp1[85] and forkhead transcription factors[86]. The Smad complex inhibits the expression of CDK4[87]. In breast epithelial cells, TGFβ also blocks cell cycle progression by decreasing the expression of CDC25 tyrosine phosphatase[87].

Repression of growth promoting transcription factors

TGFβ decreases c-MYC[76,88] by a Smad-E2F complex with p107[89], ID1/2/3[90] expression thereby inhibiting growth. Overexpression of c-MYC and ID proteins is common cancers. They regulate growth, differentiation, and angiogenesis[90-92]. Their repression downstream of TGFβ signaling participates in its anti-proliferative effects. This decreases cell growth and facilitates the induction of p15 and p21. c-MYC interacts with zinc-finger protein in both CDK inhibitor promoters, which inhibits transcription[93,94]. Inhibition of c-MYC therefore relieves both promoter regions inhibitions after TGFβ-induced transcription. The ID family of transcription factors is up-regulated in many cancers[92]. ID proteins also interact with retinoblastoma, which promotes proliferation[91]. Through Smad3, TGFβ induces expression of transcription factors[95], which repress ID1[90]. TGFβ-induced down-regulation of c-MYC inhibits ID2[91] since c-MYC binds to the promoter and induces its transcription.

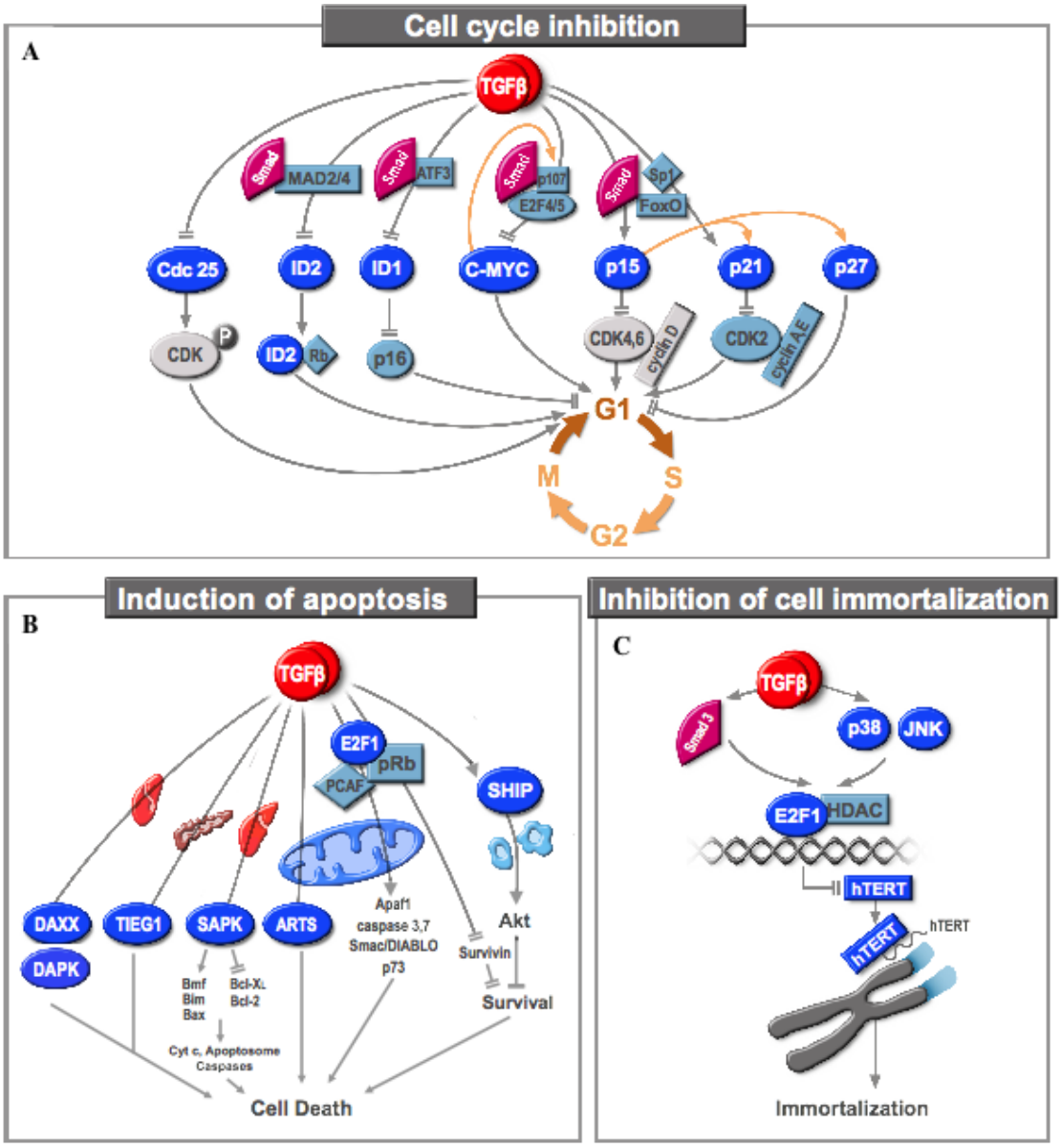


Fig. 1.9. TGFβ and tumor suppression.

(Adapted from Neel *et al.*, 2012[54])

A, Cell cycle inhibition. TGFβ exerts strong cytostatic effects and induces cell cycle arrest in the G1 phase by increasing the expression of the small cyclin-dependent kinase inhibitors p15, p21, and p27. These effects are Smad-dependent but also require the transcription factors Sp1 and FoxO. p15 directly inhibits CDK4/6 and displaces p21 and p27 from their preexisting CDK4/6 complexes allowing them to bind and inhibit CDK2-cyclin A/E complexes (orange arrows). TGFβ-induced cell cycle arrest also relies on the down-regulation of the oncogene c-myc through Smads and repressor E2F4/5. The transcription factors from the ID family are also repressed by TGFβ through Smads, MAD2/4, and ATF3, further contributing to TGFβ-mediated cell cycle arrest. Finally, other pathways, potentially more tissue specific, have been described, including upregulation of the tumor suppressor menin in pituitary adenomas, leading to G1

arrest, and down-regulation of the tyrosine phosphatase CDC25A in mammary epithelial cells, also leading to G1 arrest. **B**, Induction of apoptosis. A central pathway in the mediation of the TGFβ pro-apoptotic effects involves the E2F1-pRb-P/CAF pathway that leads to gene transcription of multiple TGFβ pro-apoptotic target genes in various types of normal and cancer cells. In hematopoietic cells, TGFβ specifically induces expression of the lipid phosphatase SHIP, which in turn decreases second messenger PIP3 level and blocks Akt-mediated survival pathways, leading to cell death in both B and T lymphocytes. Other tissue specific pro-apoptotic pathways have been described downstream of TGFβ, including the TGFβ-mediated induction of the two pro-apoptotic proteins DAXX and DAPK in liver cells, the transcription factor TIEG1 in pancreatic cells, and the mitochondrial protein ARTS. TGFβ also promotes apoptosis in an SAPK-dependent manner by inducing pro-apoptotic target gene expression (Bmf, Bim and Bax) and by repressing anti-apoptotic gene expression (Bcl-Xl and Bcl-2), further inducing mitochondrial release of cytochrome C and activation of the apoptosome, leading to caspase-dependent apoptosis in hepatocytes and B-lymphocytes. In colon cancer, TGFβ was also shown to inhibit expression of the pro-survival protein survivin. **C**, Inhibition of cell immortalization. TGFβ also exerts its tumor suppressive effects through inhibition of cell immortalization in normal and cancer cells. This effect is mediated through the Smad, p38, and JNK pathways and requires recruitment of histone deacetylases (HDAC) to the telomerase (hTERT) gene promoter, further leading to inhibition of telomerase expression, and thereby preventing cell immortalization.

Induction of apoptosis

TGFβ induces cell cycle arrest but also apoptosis in many cell types although the details are still to be understood[96-98]. Proteins such as Daax adaptor protein, TGFβ-inducible early-response gene (TIEG1) transcription factor[99], pro-apoptotic death-associated protein kinase (DAPK)[95], and mitochondrial ARTS (apoptosis-related protein in the TGFβ signaling pathway)[100] have been implicated in different TGFβ-mediated apoptosis studies. These different proteins all connect TGFβ to cell death machinery modulating BCL2 family and caspases expressions and activity[101]. TGFβ mediates apoptosis through Smad3/4[102,103] and activator protein AP1[104]. The stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signaling pathway is important downstream of TGFβ[102] as it induces pro-apoptotic Bmf and Bim from the Bcl-2 family. They then activate pro-apoptotic factor Bax, which leads to release of cytochrome c from mitochondria and apoptosome activation. This is followed by caspase-dependent apoptosis[105,106]. In colon cancer, TGFβ affects cellular survival by inhibiting pro-survival survivin through Smad3-Akt interaction[107]. In hematopoietic cells, our team has shown that TGFβ induces Src homology 2 domain-containing 5' inositol phosphatase (SHIP) expression in a Smad-dependent manner leading to Akt survival pathway inhibition and resulting in lymphocytes cell death[108]. This immunosuppressive effect of TGFβ leads to tumor progression. Our team also showed the E2F1 transcription factor to be a TGFβ target gene involved in the transcriptional complex E2F1-pRb-P/CAF for pro-apoptotic genes[109].

Prevention of cell immortalization

The Hayflick limit is the maximum number of cellular divisions that normal cells can undergo before senescence. Telomeres invariably shorten leading to senescence and death of the cell. Unlike normal cells, cancer cells are limited but immortalized. Reactivation of a telomerase enzymatic program allows cancers cells to avoid telomere shortening. Telomerase elongates telomeric DNA, which prevents shortening. The telomerase RNA component hTER and protein component hTERT function together. hTERT gene is repressed by TGFβ in a Smad3-E2F1-dependent manner[110,111] and involves both MAP kinase and histone deacetylases[43].

These effects taken together make TGFβ a potent tumor suppressor across a range of tissues.

1.2.3.2. Loss of tumor suppressive functions of TGFβ

The role of TGFβ as a potent tumor suppressor is further suggested by the fact that many inactivating mutations in its receptors or cytosolic transducers have been found to be an underlying cause for human cancer[11,17,112,113]. Numerous genetic or epigenetic alterations of the TGFβ signaling pathway components have been reported to disrupt TGFβ tumor suppressive effects, therefore favoring tumor progression[113]. These are often found in human cancers of various origin (Table 1)[11,17,112-114] and clearly illustrate the critical role played by the TGFβ signaling pathway in preventing tumor formation.

Alterations of both genetic information and accessibility for direct TGFβ signaling pathway molecules or indirect interacting partners alter the tumor suppressive role of TGFβ and lead to tumor development (Table 1.3). Inactivating mutations in TGFβ receptors and Smad genes have been reported and illustrate the tumor suppressor function of this cytokine and its role in cancer[17,19,78].

Mutations of TGFβ receptor genes

Mutations on both alleles of TβRII leading to a truncated protein or an inactive kinase activity were found in colon, gastric, biliary, pulmonary, ovarian, esophageal, head and

neck cancers, and gliomas[115]. These mutations are less frequent in endometrium, pancreas, liver, and breast cancers. The inactivating mutations of TβRII are more common in tumors with microsatellite instability that is due to mutations in mismatch repair genes. TβRI is also frequently altered in ovarian, metastatic breast, esophageal, pancreatic, and head and neck cancers by frameshift and missense mutations. Epigenetic alterations of the TGFβ receptor genes, such as promoter hypermethylation or even modified expression of the transcription factors controlling their expression, also lead to decreased receptor expression and altered activity[116]. *In vivo* expression of TβRII inhibited growth and anchorage-independent growth of several types of cancer in mice[117,118]. Also overexpression of a dominant-negative TβRII in skin[116], lung or mammary gland[119] increased tumorigenicity. Decreased expression of TβRII generally correlates with high tumor grade[120]. These observations suggest that the TGFβ receptors act as tumor suppressors at least at the early stages of carcinoma.

Mutations of Smad genes

The genes encoding for the Smads, especially Smad2 and 4, are also frequently mutated in human cancer, disrupting the formation of Smad complexes and the subsequent activation of transcription[121]. Mutations in the Smad genes are often due to loss of chromosome regions, deletions, frameshift mutations, nonsense and missense mutations[121]. They often occur in the domain called MH2 domain that allows the formation of the heteromeric Smad complex and the transcriptional activation. In particular Smad4 (also known as Dpc4 for deleted in pancreatic cancer) was found to be mutated or deleted in around 50% of human pancreatic cancers. Smad2 is mutated in a small set of colorectal, hepatic, and lung cancers[122,123] while Smad3 is lost in gastric cancer and T cell lymphoblastic leukemia[124]. Smad7 is overexpressed in pancreatic[125], endometrial [126], thyroid follicular[127] cancers resulting in the decrease of the Smad-mediated signaling. Smad4 is much more frequently altered and is considered as a tumor suppressor. Deletions affect Smad4 gene in pancreatic cancers where it was first identified as a tumor suppressor[128]. Mutations in the Smad genes occur in more than half of sporadic colorectal cancers and in esophageal cancers[123]. The transcriptional repressors Ski and SnoN, which target the Smad proteins were found to be amplified in colorectal

and esophageal cancers but also deleted in some cases[129]. Their role as oncogenes is thus context-dependent.

Table 1.3. Mutations and deletions in the TGFβ signaling pathway.

(Adapted from Neel *et al.*, 2012[54])

While expression of TGFβ itself is often increased in human tumors, expression of the genes encoding various components of the TGFβ signaling cascade (type I and II receptors, Smad2, Smad3, and Smad4) are often mutated or deleted in human cancer. Occurrence of mutation and deletion and incidence rates in different human cancers are indicated in percentage. Loss of heterozygosity (LH) is also indicated. These figures support the involvement of TGFβ in cancer.

Molecules	Cancers
	Increased expression: breast (68%), lung (48%), pancreas (47%), esophagus (37%), stomach (23%), colon, prostate.
	Mutations/deletions: colon (28%), ovary (25%), head and neck carcinoma (21%), stomach (15%), breast (12%), lung, endometrium, liver, uterus, biliary track, glyomas.
	Mutations/deletions: ovary (30%), head and neck carcinoma (17%, LH 53%), bladder (LH 31%), prostate (25%), breast (6%), biliary track.
	Mutations/deletions: colon (8%), uterus (8%), liver, lung.
	Mutations/deletions: lymphoblastic leukemia, stomach.
	Mutations/deletions: pancreas (50%, LH 90%, deletion 30%), colon (LH 60%), stomach (LH 60%), lung (LH 56%), breast (12%, LH 30%), head and neck carcinoma (LH 40%), prostate (LH 30%), biliary track (16%), uterus (4%), bladder, œsophagus, kidney, liver, ovary

Alteration of other molecules of the TGFβ signalling pathway

Mutations in the p53 gene disrupt the cooperation between p53 and Smads that is required for the TGFβ cytostatic effects in lung cancer cells and contribute to the induction of EMT, cell invasion, and metastasis formation *in vivo*[130]. The oncogenic activation of the Ras-RAF-MAPK pathway induces the transition from TGFβ-mediated tumor suppression to tumor progression in hepatocarcinoma through the phosphorylation of Smad3 linker region preventing its activation by TβRI[131]. Moreover epigenetic alterations can contribute to this transition. Indeed, hypomethylation of the PDGFβ (platelet-derived growth factor β) gene enables TGFβ to induce glioblastoma cell proliferation[132]. DAB2 (disabled homolog 2) decreased expression contributes to breast, head and neck, and squamous vulvar cancer cells progression[133]. Six1 (Sine oculis homeobox homolog 2) overexpression in breast cancer cells induces EMT by TGFβ, contributing to metastases development[134].

These alterations in the TGFβ signaling pathway alter its tumor suppressive functions and contribute to the development of metastases, thus explaining the dual role of TGFβ.

1.2.4.3. Pro-metastatic functions of TGFβ

Interestingly, while TGFβ acts as a tumor suppressor in normal cells and early carcinoma, the TGFβ growth-inhibitory and apoptotic effects are lost during tumorigenesis. Meanwhile, other TGFβ responses prevail, unrelated to growth inhibition and favoring tumor progression and metastasis (Fig. 1.10)[19,135,136]. This has been particularly well characterized in breast cancer. TGFβ not only exerts these effects on tumor cells but also on neighboring stromal cells. Indeed, tumor cells synthetize and secrete important quantities of TGFβ and TGFβ also regulates the tumor cell ability to remodel the surrounding ECM proteins by enhancing metalloproteinase expression and plasmin generation, which in turn leads to enhanced activation of TGFβ and degradation of the ECM with a consequent release of stored TGFβ. These increasing TGFβ levels modify the stroma and the ECM, stimulate tumor angiogenesis, myofibroblast differentiation, and cause local and systemic immunosuppression, further contributing to tumor progression

and metastasis. In tumor cells, TGFβ inhibits adhesion, induces EMT, promotes cell migration and invasion, and induces chemotaxis to distant organs, in turn contributing to metastasis development.

Effect of TGFβ on the tumor microenvironment (stroma)

The ECM is a TGFβ reservoir. TGFβ-induced matrix metalloproteinase (MMP) activity leads to ECM degradation, resulting TGFβ release, which in turn lead to ECM synthesis and pro-angiogenic fibroblast chemoattraction. This series of events generates a tumor-favorable microenvironment[19]. Immune surveillance escape and increased angiogenesis are essential for tumor growth.

Immunosuppressive effect of TGFβ: In healthy tissue, TGFβ acts as an anti-inflammatory cytokine and the increased expression of TGFβ largely contributes to immunosurveillance escape. Our laboratory has previously shown that TGFβ exerts profound pro-apoptotic effects in immune cells by up-regulating the lipid phosphatase SHIP[108]. TGFβ inhibits proliferation and differentiation of a range of immune cells[137] likely through inhibition of interleukin-2 and its receptors[109]. In tumor cells, TGFβ decreases expression of major histocompatibility complex class II rendering cells less immunogenic[138,139].

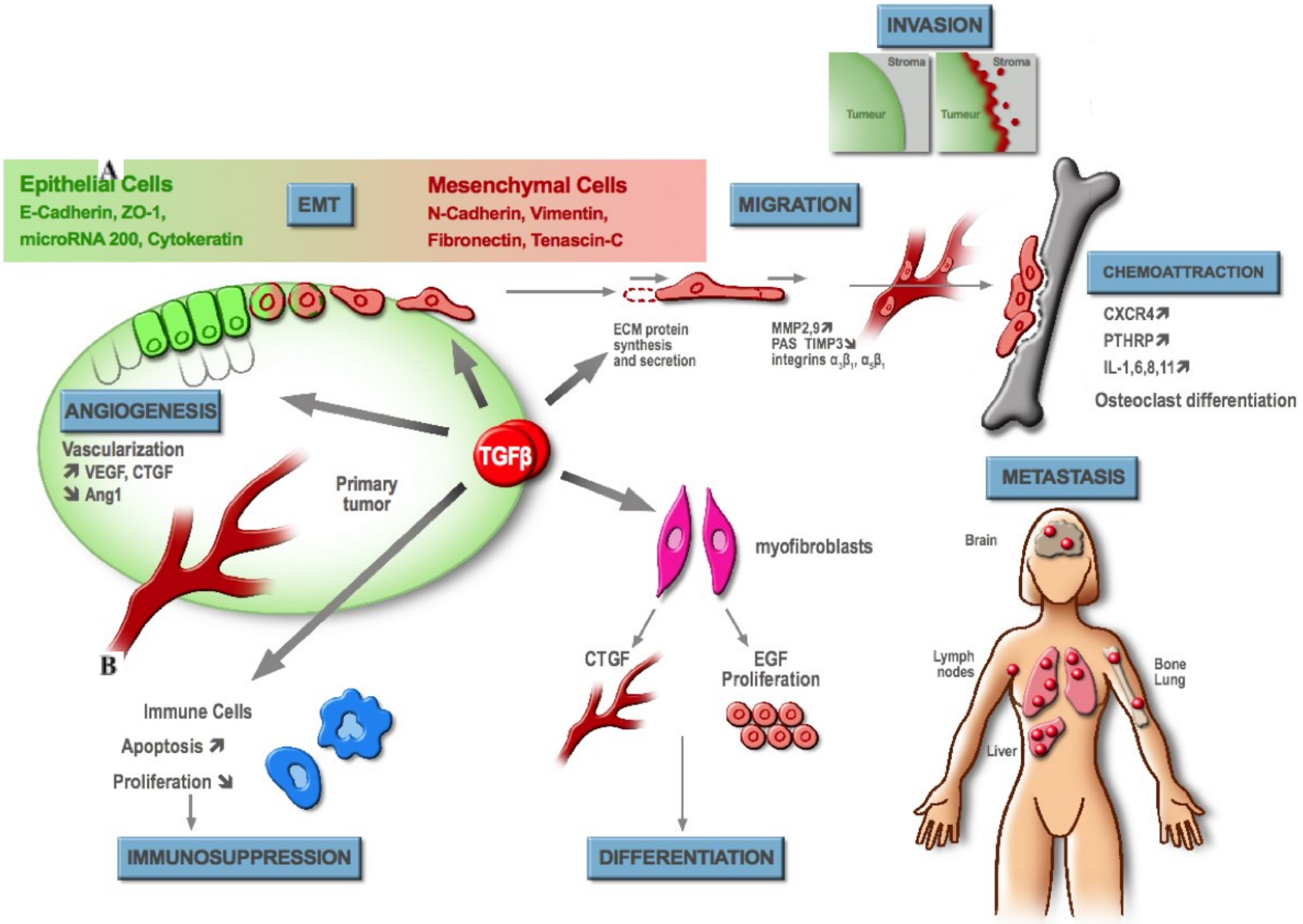
Angiogenic effect of TGFβ: Increased expression of TGFβ correlates with increased microvessel density and poor prognosis in a variety of tumor types suggesting that TGFβ is pro-angiogenic[140-142]. VEGF and CTGF are TGFβ-induced in epithelial cells and fibroblasts[143,144] and stimulate proliferation and migration. Vessel integrity maintaining Angiopoietin-1 is suppressed by TGFβ in fibroblasts[143-145] leading to leaky properties of tumor-associated blood vessels causing incomplete oxygenation of tumor areas. TGFβ-induced secretion of MMP dissolves the basement membrane to, which epithelial cells are anchored, thereby allows them to migrate and invade locally[146]. Tumor-derived myeloid immune suppressor cells (MISC) were represent about 5% of the tumor and cause further growth. These MISC release ECM-degrading and TGFβ-releasing MMP9, which in turn lead to angiogenic VEGF production. The MISC cells are incorporated into the tumor endothelium contributing to vasculature growth[147].

Myofibroblast generative effect of TGFβ: Myofibroblasts or cancer-associated fibroblasts are fibroblast-like mesenchymal cells, which resemble smooth muscle cells[148]. They accumulate in tumors early in cancer development and secrete pro-invasive and pro-angiogenic factors. TGFβ stimulates the maturation of myofibroblasts from precursor cells.

Fig. 1.10. TGFβ prometastatic effects.

(Adapted from Neel *et al.*, 2012[54])

Tumor cells secrete a significant amount of TGFβ, which affects both the cancer cell and the stroma. The autocrine and paracrine effects of TGFβ lead to tumor progression and metastasis **A**, In cancer cells, TGFβ promotes the epithelial-mesenchymal transition (EMT) by decreasing cell adhesion and blocking expression of epithelial proteins (E-cadherin, ZO-1, etc.) while increasing the expression of mesenchymal proteins (N-cadherin, vimentin, fibronectin, tenascin-C). TGFβ also promotes cell migration and invasion through multiple signaling pathways (microRNA regulation, increased synthesis and secretion of metalloproteinase expression, activation of RhoGTPases, decreased TIMP3 expression, and regulation of the plasminogen activator system (PAS)). TGFβ also promotes tumor metastasis by potentiating chemoattraction of the cancer cells to distant organs (bone, lymph node, lung, liver, and brain) and by increasing expression of cytokines (CXCR4, IL-11 and PTHrP) that will promote osteoclast differentiation and the development of osteolytic lesions. **B**, TGFβ induces angiogenesis and stimulates the vascularization surrounding the tumor by increasing VEGF and CTGF expression in epithelial cells and fibroblasts. TGFβ also inhibits expression of angiopoetin-1 in fibroblasts, thus increasing permeability of blood vessels associated to the tumor. By inducing hematopoietic cell death, TGFβ induces local and systemic immunosuppression, preventing the immune cells from infiltrating the tumor and allowing the tumor to escape host immunosurveillance. TGFβ also promotes myofibroblast differentiation, further promoting tumor growth.


INVASION
Stroma
Tumeur
Stroma
Tumeur
A
Epithelial Cells
E-Cadherin, ZO-1,
microRNA 200, Cytokeratin
EMT
Mesenchymal Cells
N-Cadherin, Vimentin,
Fibronectin, Tenascin-C
MIGRATION
CHEMOATTRACTION
CXCR4↗
PTHRP↗
IL-1,6,8,11↗
Osteoclast differentiation
ECM protein
synthesis
and secretion
MMP2,9↗
PAS TIMP3↘
integrins α3β1, α5β1
ANGIOGENESIS
Vascularization
↗ VEGF, CTGF
↘ Ang1
TGFβ
Primary
tumor
B
myofibroblasts
METASTASIS
Brain
Lymph
nodes
Bone
Lung
Liver
CTGF
EGF
Proliferation
Immune Cells
Apoptosis ↗
Proliferation ↘
IMMUNOSUPPRESSION
DIFFERENTIATION

Autocrine effect of TGFβ

TGFβ produced by the tumor cells acts on these, by stimulating their migratory and invasive properties, as well as by inducing EMT and chemotaxis.

Epithelial-Mesenchymal Transition: In order to migrate and invade, cells must dissociate from one another and acquire mesenchymal features. This epithelial-mesenchymal transition (EMT)[149,150] occurs during early developmental morphogenesis in all multicellular organisms. This is a trans-differentiation of a highly organized and tightly connected web of epithelial cells into a disorganized motile population of mesenchymal cells with stem cell-like properties. Tight junctions are lost followed by dissolution of basolateral adherens junctions resulting in loss of epithelial cell polarity marked by E-cadherin loss. Actin cytoskeleton reorganization into actin stress fibers anchored to focal adhesion complexes takes place and allow for migration. Cell-junction free cells are able to migrate. Additionally, E-cadherin down-regulation releases β-catenin from the intracellular side of the plasma membrane. It translocates to the nucleus and induces c-MYC, cyclin D1, and MMP7 expression, which favors invasion. As mentioned above, increased extracellular proteases coupled to reduced ECM proteins synthesis further favors invasion. Gradually epithelial marker expression is replaced by mesenchymal markers such as vimentin, N-cadherin, fibronectin and tenascin. The EMT process is reversibly induced by TGFβ[151,152] and is controlled by transcription factors such as Snail, Slug, Twist, ZEB-1/2, and FoxC3[17]. In vivo, blocking TGFβ signaling by the expressing a dominant-negative TβRII prevented squamous skin cancer cells from undergoing EMT[153]. During the invasive process, cells located at the TGFβ rich tumor front exhibited EMT characteristics. TGFβ-induced EMT is not only Smad-dependent, other pathways like PI3K/AKT, RhoA, and p38 MAPK pathways are involved. Importantly, a novel class of small non coding RNAs have been revealed as master regulators of EMT and its maintenance[154]. Smad-mediated expression of Snail, Slug, ZEB-2, and Twist all lead to E-cadherin repression[155] and desmosomes dissociation[156]. Notably T□RII-mediated Par6 phosphorylation promotes cell junction dissolution[157]. Ras signaling also helps TGFβ-induced EMT[158].

Pro-migratory and pro-invasive effects of TGFβ: EMT is believed to occur prior to and independently from cell migration and invasion. Independently of TGFβ-induced HMGA2 expression, Snail or Twist can induce EMT. Dominant negative TβRII expression prevents TGFβ-induced EMT and inhibits migration[159] and was rescued by constitutively active TβRI over-expression, which activated PI3K and MAPK pathways. Invasive but not non-invasive hepatocellular carcinoma cells express α3β1-integrin[160]. TGFβ induces transcriptional expression of α3β1-integrin, which is of key importance in basement membrane invasion[161]. TGFβ induce hepatocellular carcinoma cell invasion through miRNA-mediated inhibition of tissue inhibitor of metalloproteinase TIMP3[162], which in turn leads to increased MMP activity. A study on mouse skin carcinogenesis models expressing recombinant TGFβ showed that TGFβ-induced EMT could be dissociated from TGFβ-induced invasive and metastatic ability. Indeed, tumors co-expressing recombinant TGFβ and dominant negative TβRII did not undergo TGFβ-induced EMT and but more aggressive metastasis compared to tumors expressing recombinant TGFβ alone were detected[163]. Interestingly, loss of membrane-bound E-cadherin is necessary for EMT but not distant metastasis. Migrating cells generate lamellipodia at the front and retracting their trailing end. The events are coordinated by Rho-family GTPases in immune cells[164]. TGFβ also induces cell migration through Rho signaling in a Smad4-dependent manner[165].

Pro-metastatic effect of TGFβ: TGFβ potently contributes to the first of many steps, which lead to metastasis[166]. Tumor cells initially infiltrate the blood stream, disseminate and reach a secondary where they extravasate out of the blood vessel and form secondary tumor called metastasis. Tissues tropism is also mediated by TGFβ with extravasation of breast cancer cells into the lung parenchyma[113] and secretion of chemotactic cytokines[167] are shown to be Smad-dependent and TGFβ induced in breast cancer[168], melanoma[169], and renal carcinoma[170]. TGFβ stimulates bone metastasis through a stimulated parathyroid hormone related protein (PTHrP) secretion, which promotes the differentiation of osteoclast precursors and bone resorption[167] and induces expression of the bone homing receptor CXCR4. Through Smad effectors[171], TGFβ also induces

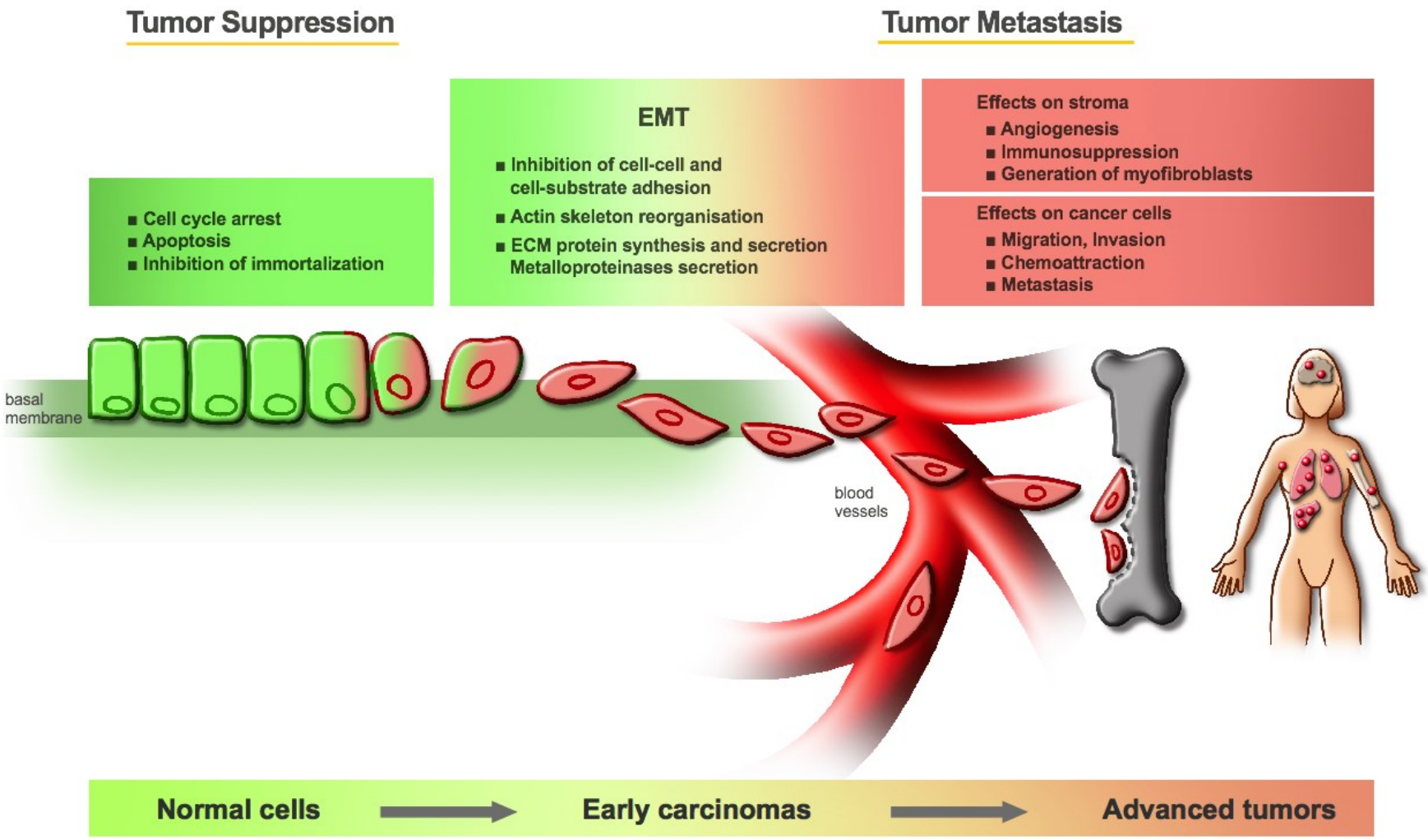
interleukin-11 and CTGF expression[90], which respectively stimulates osteoclastic factor production and mediate angiogenesis.

The involvement of TGFβ in human cancer is multifaceted (Fig. 1.11). While it initially contributes to tumor suppression by efficiently inhibiting cell proliferation, preventing cell immortalization and inducing apoptosis, TGFβ growth inhibitory responses in cancer cells are lost and invasive and pro-metastatic responses are observed as tumors progress. At this stage, TGFβ acts to promote the invasiveness of epithelial pre-malignant lesions by inducing an Epithelial-Mesenchymal Transition (EMT) and subsequently promoting metastasis. Furthermore, tumor cells produce increasing amount of TGFβ that is released in the tumor vicinity. These elevated levels of TGFβ then act on the tumor cells and the surrounding stroma to inhibit cell adhesion, induce immunosuppression, cell migration and angiogenesis and promote degradation of the ECM, further contributing to the metastatic process. Thus, TGFβ plays a major role in cancer development and progression. Thus, blocking TGFβ signaling pathway may provide for a unique therapeutic opportunity against tumor metastasis. As such, several approaches to develop new therapeutic tools that would interfere with the TGFβ pathway have been undertaken in recent years[14]. As TGFβ is a key regulator of cancer progression and particularly involved in metastasis, the pharmaceutical industry has attempted to interrupt TGFβ signaling *in vivo* in the hope of improving patient prognosis and treatment outcome. The following chapter-manuscript summarizes the various strategies that have been attempted and introduces a new type of therapeutic strategy.

Fig. 1.11.TGFβ from cancer suppression to cancer progression

(Adapted from Neel *et al.*, 2012[54])

In several types of cancer, TGFβ acts as a tumor suppressor in normal cells and early carcinomas, while it promotes tumor metastasis in more advance stages of cancer. The tumor suppressive effects of TGFβ include cell cycle arrest, apoptosis, and prevention of cell immortalization. TGFβ also induces EMT, marked by a decrease in cell-cell and cell-substrate contacts, a reorganization of the actin cytoskeleton, as well as an increase in MMP synthesis and secretion. In human cancers, inactivating mutations in the TGFβ signaling components or activating mutations in oncogenic signaling pathways are often observed and provide an underlying basis for tumor development. These mutations attenuate the TGFβ tumor suppressive effects but do not affect its tumor promoting effects on cancer cells and on the surrounding environment, including EMT, cell migration and invasion, angiogenesis, immunosuppression, myofibroblast generation, chemoattraction, and tumor metastasis, further promoting TGFβ-induced tumor progression to secondary distant sites.


Tumor Suppression
Tumor Metastasis
EMT
Cell cycle arrest
Apoptosis
Inhibition of immortalization
Inhibition of cell-cell and cell-substrate adhesion
Actin skeleton reorganisation
ECM protein synthesis and secretion Metalloproteinases secretion
Effects on stroma
Angiogenesis
Immunosuppression
Generation of myofibroblasts
Effects on cancer cells
Migration, Invasion
Chemoattraction
Metastasis
basal membrane
blood vessels
Normal cells
Early carcinomas
Advanced tumors

1.3.Targeting TGFβ signalling in human cancer therapy

(Published in Trends in Cell and Molecular Biology, 2010 (5),69-107)

As described above, while the tumor suppressor effects of TGFβ are lost in aggressive human tumors, TGFβ signaling is still active in these cancer cells and promotes invasive and pro-metastatic responses. Thus, targeting the TGFβ signaling pathway provide for a unique therapeutic opportunity against tumor metastasis. As a result, many efforts have been made in recent years to develop therapeutic tools that would interfere with this pathway. Three main approaches have been defined to counteract the TGFβ-induced effects on cancer progression (Table 1.4). The first set aims at preventing ligand-receptor interaction using blocking monoclonal antibodies, soluble receptors and peptide inhibitors. The second group of approaches is based on blocking TGFβ production at the translational level, using antisense oligonucleotides. Finally, the last set made use of blocking the downstream receptor-mediated signaling cascade, by interfering with the receptor kinase activity, using small-molecule inhibitors. The present manuscript will review the different molecules that have been developed in the context of these three strategies.

1.3.1. Blocking ligand-receptor interaction with competition strategies

1.3.1.1.Monoclonal antibodies

Definition and use in drug design

A major problem with most treatments in the past was the lack of specificity for tumor cells, leading to low efficiency and significant side effects. The idea of using antibodies to selectively target tumors was first suggested by Paul Ehrlich at the beginning of the 20th century, but this strategy has been only really possible since 1975 with the development of the hybridoma technology for the production of monoclonal antibodies[172,173]. This very promising technology was even awarded with the Nobel Prize in 1984. The first antibodies were produced in mice, which were immunogenic in humans and did not induce significant immune responses. The production of chimeric, humanized, or fully

human monoclonal antibodies has overcome these issues. This new generation on antibodies has a longer half-life in the blood stream and is able to interact with the patient's immune system. Rituximab was the first monoclonal antibody approved for cancer therapy, for the treatment of several types of lymphoma and leukemia. Now more than 20 monoclonal antibodies have been approved by the Food and Drug Administration (FDA), of, which 6 are specific to cancer therapy (**Table 4**). The antibodies can be either unconjugated or conjugated to radioactive isotopes or chemotherapeutic drugs. Many of them target tumor cells antigens or the tumor microenvironment to enhance immune response.

Targeting TGFβ signalling pathway with monoclonal antibodies

TGFβ isoform-specific antibodies

Lerdelimumab: Lerdelimumab, or CAT-152 or Trabio (Cambridge Antibody Technology) is a recombinant human IgG4 directed against TGFβ-2 that is able to neutralize TGFβ-2 (IC_{50} = 0.9 nM) and TGFβ-3 at a lesser extent (IC_{50} = 10 nM), but not TGFβ-1 in the same range of concentrations[174]. This antibody was designed by phage display for the treatment of fibrotic disorders, by isolating a human single chain Fv fragment that neutralizes human TGFβ-2 from a phage display repertoire and converting it into a human IgG4[174]. TGFβ-2 is the most predominant isoform in the eye and is the most potent growth factor at stimulating conjunctival fibroblast function[175]. Elevated levels of TGFβ-2 are found in the aqueous of glaucomatous eyes compared with normal eyes. This led to the search for strategies that would neutralize the effects of TGFβ-2 and thus reduce conjunctival scarring after glaucoma filtration surgery. Lerdelimumab was efficient in improving surgical outcome and reducing subconjunctival collagen deposition in rabbits subjected to glaucoma surgery. Another study showed that lerdelimumab was able to attenuate fibrosis in an experimental diabetic rat kidney[176]. Phase I and II clinical trials with patients undergoing glaucoma surgery showed good tolerability and were promising[177]. However, a phase III study failed to demonstrate an improvement of the outcome of surgery for glaucoma. Lerdelimumab has therefore been discontinued[178]. A phase III trial also failed to show any improvement in preventing the failure of primary trabeculectomy[179,180]

Metelimumab: Metelimumab or CAT-192 (Cambridge Antibody Technology and Genzyme) is a human IgG4 monoclonal antibody directed against TGFβ-1 developed with the same technology as lerdelimumab. TGFβ-1 plays an important role in the fibrogenic process of diabetic nephropathy. In preclinical studies with rat model of diabetes, metelimumab in combination with the angiotensin-converting enzyme attenuated several characteristics of the disease[181]. TGFβ-1 or TGFβ-2 expression is a hallmark of early skin lesions and systemic sclerosis-associated interstitial lung disease[182]. In a phase I/II clinical trial metelimumab showed no evidence of efficacy for the treatment of systemic sclerosis[183]. It has been discontinued[178].

Pan-TGFβ antibodies

Considering that multiple TGFβ isoforms influence tumors, it is reasonable to think that antibodies against all isoforms would be more effective than isoform-specific antibodies and several attempts have been made to develop such blocking pan-antibodies.

1D11: 1D11 is a murine IgG1 monoclonal antibody developed in the late 1980s[184]. 1D11 was shown to attenuate hallmarks of diabetic nephropathy in combination with the angiotensin-converting enzyme in the same study as for metelimumab[181]. This antibody has not been further studied in clinical trials because it may generate immunogenicity in humans.

2G7: Intraperitoneal injections of 2G7 (an murine IgG2b neutralizing antibody) following intraperitoneal inoculation of MDA-MB-231 breast cancer cells in mice suppressed intraabdominal tumor and lung metastases[185]. It also resulted in a significant increase in mouse spleen natural killer cell activity. However, mice lacking natural killer activity showed no reduction in tumor growth or metastasis. This antibody was tested in sporadic clear cell renal carcinoma in, which TGFβ-1 is overexpressed[186]. It did not suppress the growth of clear cell renal carcinoma cells *in vitro* but inhibited tumorigenesis *in vivo* and even induced the regression of established tumors in athymic mice. It was hypothesized that 2G7 inhibited angiogenesis.

GC-1008: GC-1008 belongs to a series of human pan-TGFβ monoclonal antibodies (Cambridge Antibody Technology and Genzyme). GC-1008 was designed to bind with high affinity to TGFβ isoforms via a single interaction surface[187]. It is currently in phase I clinical trials for the treatment of idiopathic pulmonary fibrosis, melanoma, renal cell carcinoma, and focal segmental glomerulosclerosis. The patients with advanced malignant melanoma and renal cell carcinoma tolerated the treatment well.

Other monoclonal antibodies against TGFβ are currently being developed by Genentech[188]

1.3.1.2.Soluble receptors

Definition and use in drug design

Cytokines are involved in a variety of processes, such as immune response and haematopoiesis. Their deregulation often leads to pathologies. Thus, they have drawn attention as therapeutic targets. One interesting strategy has been developed following the discovery of soluble cytokine receptors, which efficiently inhibited the binding of their cytokine to the membrane-anchored receptors and their subsequent activity[189]. One of the first proofs of principle for soluble cytokine receptors was the soluble tumor necrosis factor (TNF) receptor. It inhibited TNF activity in a murine endotoxemia model[190]. Three soluble TNF receptors have been approved by the FDA for the treatment of rheumatoid arthritis and other chronic inflammatory diseases since 1998: etanercept, infliximab, and adalimumab[191].

Targeting TGFβ signalling pathway with soluble receptors

This approach has also been applied to the TGFβ signaling pathway. The exogenous expression of a soluble TβRII in human pancreatic cancer cell lines led to the abrogation of TGFβ1-induced growth inhibition and to a decrease in their invasive capacity *in vitro*, as well as slow-growing tumors, low angiogenesis, and low expression of plasminogen activator inhibitor 1 (PAI-1) *in vivo*[192]. This indicated that using a soluble TGFβ receptor could block tumorigenic effects of TGFβ. The same soluble receptor was also tested in pancreatic ductal adenocarcinoma that is known to overexpress TGFβ isoforms and form metastasis[193]. The cells expressing this soluble receptor showed decreased tumor growth

and weakened PAI-1 expression when subcutaneously injected into mice. When orthotopically injected, their ability to metastasize was suppressed and the expressions of PAI-1 and urokinase plasminogen activator (uPA) were decreased. This suggested the potential use of soluble TβRII as a therapeutic tool. The 159 amino acid-extracellular domain of TβRII was expressed as a recombinant protein and was able to inhibit TGFβ activities in mouse and insect cells[194]. Also recombinant soluble TβRIII, called betaglycan, strongly inhibits the binding of TGFβ isoforms to the membrane-anchored receptor and thus blocks TGFβ activities[195]. Attenuated TGFβ effects were seen with decorin, a proteoglycan induced by TGFβ[196]. Indeed, decorin attenuates extracellular matrix accumulation in glomeruli, which is hallmark of kidney disease. In order to facilitate protein purification later on, a chimeric soluble receptor was constructed by fusing the extracellular domain of TβRII to the Fc regions of human immunoglobulin IgG1 (Fc:TβRII or SR2F)[197,198]. This chimeric soluble receptor showed similar biochemical properties as the wild-type receptor. It strongly inhibited the TGFβ-induced growth inhibition and extracellular matrix formation. Fc:TβRII was also tested in transgenic mice[199]. Although tumor latency was not affected, the soluble receptor increased the apoptosis rate in primary tumors and reduced tumor cell motility, intravasation, and lung metastases. Metastases were also inhibited by Fc:TβRII in mammary tumors in syngeneic BALB/c mice. Since most patients die from metastases, SR2F is an attractive approach as an anti-cancer agent.

1.3.1.3.Peptide inhibitors

Peptide inhibitors are short peptide sequences derived from TGFβ receptors that block ligand binding to its receptors. Synthetic peptide inhibitors of TGFβ-1 to its receptors were designed as overlapping peptides encompassing the sequence of TGFβ-1 and the extracellular region of type III TGFβ-1 receptor and peptides from the TGFβ-1 type III receptor predicted to bind to TGFβ-1 according to computational predictions[200]. Efficient peptides were then selected according to their ability to reverse the TGFβ-1-induced growth inhibition. Peptides P11 and P12, derived from TGFβ-1, and P54 and P144, derived from its type III receptor, prevented TGFβ-1-dependent inhibition of proliferation

in vitro and reduced binding of TGFβ-1 to its receptors. The most advanced peptide inhibitor is P144 (DigNA Biotech), which is the acetic salt of a 14mer peptide sequence. P144 blocks the binding of TGFβ-1 to TβRI and TβRII. Intraperitoneal administration of P144 in rats showed anti-fibrogenic activity in the liver. P144 inhibited TGFβ-1-dependent signaling cascade and collagen type I synthesis in cardiac fibroblasts and potentially prevented myocardial fibrosis in spontaneously hypertensive rats[201]. It is currently tested in a phase II clinical trial for the treatment of patients with skin fibrosis in systemic sclerosis[178].

1.3.2. Blocking TGFβ production with antisense strategies

A direct way to prevent the effects of the TGFβ signaling pathway is to reduce the amount of the stimulus of the cascade, *i.e.* the TGFβ ligand.

1.3.2.1.Antisense oligonucleotides

Definition and use in drug design

Antisense oligonucleotides (ASO) have been developed for this purpose. ASO are 13-25-nt single-stranded nucleic acids that have been chemically modified or not[202,203]. Their sequence is designed to be complementary to the target mRNA. When an ASO hybridizes to its target by Watson-Crick base pairing, the mRNA-ASO duplex is recognized by RNase H enzymes, which cleave the mRNA strand, leaving the ASO intact. These released ASO can then bind to other mRNAs. Besides this main process, ASO can inhibit their mRNA target function by modulation of splicing, inhibition of protein translation by disruption of ribosome assembly, and disruption of necessary three-dimensional structure. These processes are highly specific since highly homologous genes may be inhibited selectively[204]. The specificity of this strategy raised interest for therapeutic applications. Unlike usual drugs for, which the exact mechanism of action is unknown or poorly characterized, the target-specificity of ASO makes them essential tools for therapy. This led to intense research on ASO drug design. The first *in vitro* experiments blocked the replication of Rous Sarcoma Virus in infected chicken embryo fibroblasts[205]. One of the first studies on the *in vivo* activity of ASO showed that targeting N-Myc with an oligonucleotide delivered in the vicinity of a neuroepithelioma murine tumor by a

subcutaneously implanted micro-osmotic pump led to a loss of N-MYC protein and a decrease in tumor mass[206]. Many studies then followed on other targets. However, due to problems of efficiency and selectivity chemical modifications of ASO have been developed.

Chemical modifications

The initial ASO showed lack of potency and selectivity[207]. Secondary structures in RNA decrease accessibility to ASO. Since these structures are hard to predict, many ASO typically need to be tested. ASO doses were often inducing nonselective toxicity and cell death. The ability of ASO to bind to proteins led to artificial effects. Finally ASO were highly susceptible to degradation by nucleases. Chemical modifications have been developed to circumvent these issues. Phosphorothioate (PS) linkages provided the first generation of chemically modified ASO[208] with nuclease resistance thereby slowing their degradation and increasing their *in vivo* half-life by promoting binding to serum proteins. This high affinity for proteins also caused artifactual phenotypes. Further modifications led to the second generation of modified ASO. 2'-modified RNA cannot be cleaved by RNase and a six-base DNA fragment is necessary in the ASO to induce the cleavage of the mRNA target. These modifications led to the development of several ASO types: peptide nucleic acids (PNA), 2'-O-methoxyethyl RNA, morpholino, and locked nucleic acid (LNA).

Targeting TGFβ signalling pathway with ASO

AP 11014: AP 11014 is a PS-ASO designed to target TGFβ-1 mRNA. This TGFβ isoform is highly expressed in non-small cell lung cancer, colon cancer, and prostate cancer. This compound significantly reduced TGFβ-1 secretion in non-small cell lung cancer, colon cancer, and prostate cancer cell lines[209]. It also induced an inhibition of tumor cell proliferation, as well as a reduced migration in non-small cell lung cancer and prostate cancer *in vitro* and it finally reversed the TGF-β1-induced immunosuppression[210]. AP 11014 is currently in advanced preclinical studies.

AP 12009: also known as trabedersen (Antisense Pharma), is an 18-mer PS-modified ASO that was designed to target the TGFβ-2 mRNA in TGFβ-2 overexpressing tumors,

such as malignant gliomas, pancreatic carcinomas, metastatic colorectal carcinomas and malignant melanomas. AP 12009 was first designed for malignant gliomas. Indeed, these tumors are often associated with depression of immune responsiveness, particularly with low numbers of circulating T lymphocytes[211]. Overexpressed TGFβ-2 in these tumors has been implicated in immune response regulation, for example depression of T cell-mediated tumor cytotoxicity[212]. Targeting TGFβ-2 with AP 12009 significantly decreased TGFβ-2 secretion levels[213,214]. It also significantly decreased proliferation and spheroid migration of glioma and pancreatic carcinoma cells. Finally it enhanced lymphocyte proliferation and autologous tumor cytotoxicity. AP 12009 showed higher efficacy and cellular uptake without carrier liposomes, as well as lower non-specificity, amongst other ASO designed for the same target gene. In those studies, AP 12009 also showed a higher efficacy than monoclonal anti- TGFβ-2 antibodies. AP 12009 also showed efficacy in pancreatic cancer[188,209]. AP 12009 was assessed in three phase I/II open-label dose escalation studies for the treatment of recurrent or refractory high-grade glioma (anaplastic astrocytoma, WHO grade III) and glioblastoma (WHO grade IV)[213,214]. In patients with high-grade glioma, the compound was delivered throughout the brain tumor tissue and the area surrounding the tumor by convection-enhanced delivery, allowing AP 12009 to bypass the blood–brain barrier and ensuring direct delivery to the tumor. AP 12009 was shown to be safe and well-tolerated and few adverse events were observed. The studies demonstrated median overall survival benefit of 17.4 months and long-term tumor responses compared with standard temozolomide chemotherapy. A phase III trial has started in 2009 with patients with recurrent or refractory anaplastic astrocytoma (WH grade III). Phase I/II studies for pancreatic carcinoma and malignant melanoma are currently ongoing[215].

1.3.2.2.TGFβ vaccine

Belagenpumatucel-L (NovaRx) is a nonviral gene-based allogeneic tumor cell vaccine[216]. This vaccine consists of genetically modified syngeneic or allogeneic tumor cells expressing antisense DNA to TGFβ-2[217]. In preclinical studies, the vector was generated by inserting a 932-base pair fragment of the TGFβ-2 gene in reverse orientation in a plasmid vector under the cytomegalovirus promoter. Genetic modification of the rat

gliosarcoma cells was performed by electroporation followed by selection of pools of clones. Rats with established intracranial gliomas were then immunized subcutaneously with the modified gliosarcoma cells. The treated animals survived for the period of the study, whereas only one seventh of the control animals survived. However, in a murine ovarian teratoma model, the antisense therapy failed to induce significant protection but significantly improved performance when combined with interleukin-2 treatment[218]. In a phase I clinical study, patients with astrocytoma (WHO grade IV) received subcutaneous injections of autologous tumor cells containing the TGFβ-2 antisense vector[216]. The treatment was well tolerated. The TGFβ-2 secretion by tumor cells was greatly reduced. The overall median survival was longer than with conventional therapy (78 weeks versus 47 weeks). The vaccine had apparently induced humoral and cellular immunity. A phase II clinical trial was conducted with non–small-cell lung cancer patients[219]. Again the treatment was well tolerated. A dose-related survival difference was observed. Indeed, the estimated probabilities of 1-year and 2-year survivals were significantly higher in the high-dose group compared to the low-dose group. The patients also demonstrated an increase cytokine production, indicating that the vaccine was efficient at reducing the TGFβ-2 induced immunosuppression. Another phase II study also proved high tolerability and safety of the treatment in non-small cell lung cancer patients[220]. A phase III trial is going to be conducted with patients with advanced non-small cell lung cancer[221].

1.3.3. Inhibiting the receptor-medicated signalling cascade with kinase inhibitors

The last strategy consists of targeting the initiation of the signaling cascade using small-molecule kinase inhibitors. Peptide aptamers targeting the Smad proteins have been designed but their development is very limited[222].

Strategy	Molecule	Target(s)	Company	Stage(s), disease(s)
BLOCKING LIGAND-RECEPTOR INTERACTION				
Monoclonal antibodies	Lerdelimumab (CAT-152, Trabio)	TGFβ-2	Cambridge Antibody Technology	Phase III, glaucoma: discontinued
	Metelimumab (CAT-192)	TGFβ-1	Cambridge Antibody Technology and Genzyme	Phase I/II, systemic sclerosis: discontinued
	1D11	TGFβ-1, 2, 3	Genzyme	-
	2G7	TGFβ-1, 2, 3	Genentech	-
	GC-1008	TGFβ-1, 2, 3	Genzyme	Phase I, kidney sclerosis Phase I/II, solid tumors Phase I/II, pulmonary fibrosis
Soluble receptors	SR2F	TGFβ-1, 2, 3	NCI/NIH	-
Peptide inhibitors	P144 (Disitertide)	TGFβ-1	DigNA Biotech and ISDIN	Phase II, systemic sclerosis
BLOCKING TGFβ PRODUCTION				
Antisense oligonucleotides	AP 11014	TGFβ-1 mRNA	Antisense Pharma	-
	AP 12009 (Trabedersen)	TGFβ-2 mRNA	Antisense Pharma	Phase III, anaplastic astrocytoma Phase I/II: pancreatic carcinoma and malignant melanoma
TGFβ vaccine	Belagenpumatucel-L (Lucanix)	TGFβ-2 mRNA	NovaRx	Phase III, advanced non-small cell lung cancer
BLOCKING THE RECEPTOR-MEDIATED SIGNALING CASCADE				
Small-molecule kinase inhibitors	LY580276	TβR1	Eli Lilly Research	-
	LY364947	TβR1	Eli Lilly Research	-
	LY2157299	TβR1	Eli Lilly Research	Phase I, metastatic malignancies
	SD-093	TβR1	Scios	-
	LY573636	TβR1	Eli Lilly Research	Phase II, malignant melanoma, soft tissue sarcoma, non-small cell lung and ovarian cancer
	A-83-01	TβR1	Kyoto Pharma	-
	SB-505124	TβR1	GlaxoSmithKline	-
	SD-208	TβR1	Scios	-
	SX-007	TβR1	Scios	-
	IN-1130	TβR1	In2Gen Co.	-

Table 1.4: Three strategies to target TGFβ signalling in therapy (From Humbert *et al.* 2010[14]) TGFβ signalling can be targeted at a free ligand level, at the receptor level or at the intracellular transduction level thereby effectively blocking biological effects

	SB-431542	TβR1	GlaxoSmithKline	-
	NPC-30345	TβR1	Scios	-
	NPC-30485	TβR1	Scios	-
	LY2109761	TβR1, TβRII	Eli Lilly Research	-
	LY294002	TβR1, P13K	Eli Lilly Research	-

Table 1.4 continued: Three strategies to target TGFβ signalling in therapy

1.3.3.1.Definition and use in drug design

The previous strategies involve large-molecule inhibitors of the TGFβ pathway that target the ligand itself. Another possibility is to prevent the receptor from initiating the signaling cascade upon ligand stimulation. It is in this prospect that small-molecule kinase inhibitors have been developed. Efforts have focused on TβRI because of the extensive knowledge of the effects of the phosphorylated Smad proteins and because targeting TβRI would not disrupt potential TβRI-independent pathways initiated by TβRII[177,178]. Four main types of kinase inhibitors exist: the classical pyrazole-based, imidazole-based, triazole-based, and the non-classical inhibitors[178]. The TβRI kinase inhibitors harbor specific features, such as a 2-pyridyl group in the hydrophobic pocket implicated in a water mediated hydrogen bond network, and a substituted aromatic group or an heteroaryl group that acts as an hydrogen-bond acceptor[178]. These warhead groups have the ability to form a specific hydrogen bond to the ATP-binding site on the receptor. This kinase domain is very different between TβRI and TβRII implying a higher specificity of the inhibitors. The major approach to identify novel kinase inhibitors consists of the biological screening of chemical collections and natural products.

1.3.3.2.TGFβ type I receptor kinase inhibitors

A library of potential TβRI kinase inhibitors was screened in a cell-based assay and tested as inhibitors of autophosphorylation of a human TβRI domain that is constitutively active[223-225]. They were also tested as inhibitors of TGFβ-induced transcription with a luciferase reporter assay in mink lung cells and growth in mouse fibroblasts. LY580276[224] and LY364947/HTS466284[226] were amongst the most potent inhibitors in the library. Several of these selected compounds were tested in xenograft models in breast and non-small cell lung cancers and showed tumor growth delay *in vivo*[227]. Oral administration of LY2157299 with advanced malignancies was safe and well tolerated in a phase I clinical trial[228]. Another inhibitor, SD-093, strongly altered the *in vitro* motility and invasiveness of pancreatic carcinoma cells[229]. It also inhibited the TGFβ-induced migratory and invasive abilities in Smad4 deficient pancreatic cancer cells[229]. This

compound also inhibited EMT induced by TGFβ in a normal murine mammary epithelial cell line[230,231]. LY573636 is being tested for the treatment of patients with malignant melanoma, soft tissue sarcoma, non-small cell lung cancer and ovarian cancer[223,232]. The inhibitor A-83-01 was found to inhibit the transcriptional activity induced by TβRI[233]. It also inhibited the transcriptional activity induced by the activin type IB and nodal type I receptor whose kinase domains are structurally very similar to that of TβRI. A-83-01 inhibited the TGFβ-induced EMT. SB-505124 is able to block activin A receptor in addition to TβRI[234]. It was shown to inhibit TGFβ-induced activation of MAP kinase pathways and to block TGFβ-induced cell death in a rat hepatoma cell line. SD-208 inhibited the TGFβ-induced growth of murine glioma cells[235]. It blocked the autocrine and paracrine TGFβ signaling and inhibited TGFβ-induced migration and invasion. It also increased the infiltration of immune effector cells into murine glioma tumors and prolonged survival[235]. SX-007 showed the same effects in a murine *in vivo* glioma model[236]. SD-208 down-regulated cytokine secretion mediating multiple myeloma cell growth[237]. SD-208 also inhibited pulmonary fibrosis at the time of initiation of fibrogenesis and blocked progressive fibrosis in animals with established fibrosis[238]. In a non-cancer model, IN-1130 reduced levels of TGFβ-1 and type I collagen mRNAs, inhibited TβRI-mediated Smad2 phosphorylation and blocked renal fibrogenesis induced by unilateral ureteral obstruction in rats[239]. SB-431542 is a selective inhibitor of Smad3 phosphorylation that inhibits TGFβ-1-induced nuclear Smad3 localization[240]. It also inhibited TGFβ-1-induced fibronectin and type I collagen synthesis in renal epithelial carcinoma cells. NPC-30345 and NPC-30488 were able to inhibit TGFβ-induced Smad2 phosphorylation as well as TGFβ-induced transcription of a luciferase reporter in mink lung epithelial cells[230].

1.3.3.3.Other kinase inhibitors

LY2109761 is a TGFβ dual receptor kinase inhibitor that is able to inhibit TGFβ-mediated activation of Smad and non-Smad pathways in colon adenocarcinoma cells having K-Ras mutation and to attenuate the oncogenic effects of TGFβ on cell migration, invasion and tumorigenicity[241]. It decreased liver metastases and prolonged survival in a

murine pancreatic cancer model[242]. It was also shown to block migration and invasion of hepatocellular carcinoma cells by up-regulating E-cadherin[243]. LY294002 is a PI3 kinase inhibitor and was shown to block the suppression of TGFβ-mediated apoptosis in transformed cells[244]. To date there is very little clinical data available for small-molecule kinase inhibitors of TGFβ receptors[223]. The different strategies used to target TGFβ are briefly summarized in Fig.1.12.

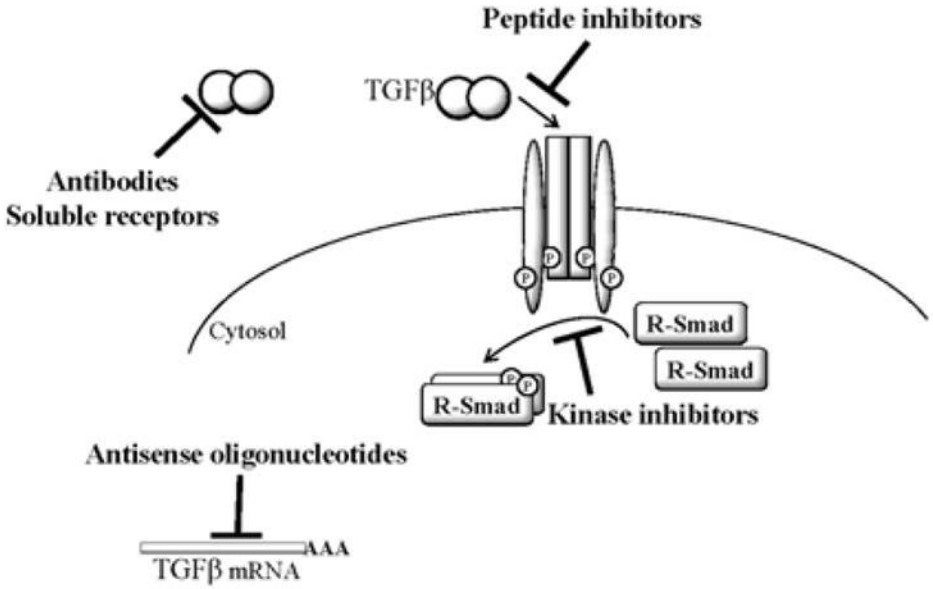


Fig. 1.12: Summary of TGFβ signaling pathway inhibition strategies (From Humbert et al. 2010[14])

Therapeutic strategies targeting TGFβ block the free ligand (monoclonal antibodies, soluble receptors), the ligand-receptor interactions (peptide inhibitors), the receptor kinase activity (small-molecule kinase inhibitors), or the production of the ligand (antisense oligonucleotides). Antisense strategies required efficient intracellular delivery of the therapeutic agent.

1.3.4. The challenges of targeting TGFβ in human cancer therapy

The three approaches to develop TGFβ inhibitors have been successful and several molecules are currently being clinically investigated. In addition to potential lacks of efficiency, each type of therapy has advantages and disadvantages[188].

1.3.4.1.Physical limitations of targeting TGFβ

The three strategies are limited by physical barriers. Indeed, they have to penetrate several barriers to reach the tumor: the vascular endothelium, stromal barriers, high

interstitial pressure, and epithelial barriers. For instance the antisense strategy has limited organ and tissue penetration. The convection-enhanced delivery counteracts this limitation and ensures direct delivery to the tumor[214].

1.3.4.2.Specificity of the TGFβ signalling pathway

One of the main concerns in targeted therapy is the off-target effects. Indeed the molecules may be able to target molecules implicated in other important signaling cascades and induce responses that do not participate in the tumor suppression but can even promote tumor growth. The antisense strategy is highly specific since it targets specific mRNA sequences and since the recognition needs to be perfect to induce mRNA degradation. However, as mismatches are tolerated in RNA interference, it is probable that ASO affect other mRNAs at the translational level. Small-molecule kinase inhibitors are not as specific. Indeed a large group of protein kinase inhibitors was tested for their ability to inhibit non-specific proteins and it was shown that even the most selective inhibitors affected at least one other protein kinase[245].

1.3.4.3.Potential risks due to TGFβ targeting

As previously mentioned, TGFβ has a dual role in cancer. Targeting TGFβ mainly deals with the pro-metastatic arm. However, this is only beneficial if the tumor suppressor arm is not affected. TGFβ-1 disruption by genetic recombination in mouse was shown to lead to multi-organ failure as a result of inflammation[246]. This phenotype suggests that total disruption of TGFβ signaling can lead to systemic inflammation and autoimmune phenomena. However, lifetime exposure to TGFβ antagonist had no major effects in mice models indicating that residual TGFβ activity was sufficient to avoid systemic inflammation[198]. To date, exogenous TGFβ has not been shown to inhibit tumor growth *in vivo* nor has the administration of antagonists been shown to neither induce tumors nor accelerate the growth of existing tumors[247]. Indeed, several studies showed that a decrease in TGFβ signaling can enhance tumorigenesis[119,248-251]. Also TGFβ is a potent growth inhibitor and is involved in protecting the genome from cytogenetic abnormalities. In that sense, targeting TGFβ could therefore accelerate proliferation and decrease genome stability leading to selective outgrowth of aggressive cells[252].

TGFβ signaling plays an important role in pancreatic cancer as indicated by the fact that 55% of patients have mutated Smad4. Although pancreas-specific TβRII knock-out mice have no discernable phenotype after 1.5 years when combined with additional pancreas-specific constitutively active K-ras oncogene, which alone induces only intraepithelial neoplasia, the median survival dramatically drops to 59 days. This indicated that TGFβ signaling functions as a tumor suppressor in this genetic context[253]. In breast cancer, the role of TGFβsignaling is complex. A breast-specific TβRII knock-out in a viral oncogene-driven metastatic cancer model leads to decreased tumor latency, increased apoptosis and increased pulmonary metastasis. This indicated that TGFβ signaling functions as a tumor suppressor in this context as well[254]. In a Neu-driven breast cancer model, constitutively active TβRI increased the latency of the primary mammary tumor but also increased pulmonary metastasis whereas a dominant negative TβRII decreased the latency of the primary tumor but also significantly decreased the number of lung metastases[113]. This finding indicates that although TGFβ acts as a tumor suppressor on the primary tumor, it may act on the ability of the breast cancer cells to extravasate from lung vessels to the parenchyma. Although loss of TβRII correlates with poor prognosis in esophageal cancer[255] and renal carcinoma[256] it also correlates with better survival rate in colon cancer[257] and gastric cancer[258] clearly indicating that the role of TβRII in carcinogenesis may be stage and tissue specific. Constitutively active TβRI signals through the Akt pathway leading to increased survival. Neu-driven tumors are more metastatic in the presence of active TβRI indicating that TGFβ signaling provides a gain-of-function advantage[259]. A study on 460 patients with high grade colon cancer where TβRII is often lost due to microsatellite instability revealed that the retention of functional TβRII decreased the five-year survival from 74% to 46%. This finding indicated that targeting TGFβ could be beneficial in this context[257]. Overall, it seems that the beneficial effects of TGFβ could be context-dependent. These potential risks give rise to the necessity to identify patients in, which the pro-metastatic arm of the TGFβ signaling pathway is predominant. Assessing the levels of TGFβ in the serum or the tumor has been studied as a tool for screening patients, showing a correlation between high serum levels and tumor progression and metastasis[188]. Many efforts have been made to target TGFβ in cancer due to its crucial role in cancer progression. Several strategies

have been developed and some molecules have shown encouraging and promising results. However, these strategies have still very important challenges to overcome. Alternatively, new strategies may be developed in the future. One such hopeful candidate may be revealed by the recent discovery of a new class of small nucleic acid molecules, the microRNAs.

1.4.MicroRNA and human cancer

1.4.1. microRNA discovery, biogenesis and function

1.4.1.1.microRNA discovery

MicroRNA (miRNAs) have eluded researchers for decades stealthily regulating many of the major biological processes in eukaryotic cells. In the past decade, our understanding of miRNA has grown tremendously from an observed oddity in worms to the establishment of a fully recognized new class of regulatory molecules with over a thousand members so far. They are a novel class of small (19-25nt) non-coding RNAs, which play important roles in development. miRNAs have been shown to regulate gene expression post-transcriptionally by guiding the RNA-induced silencing complex (RISC) to their cognate site of the 3'untranslated region (3'UTR) of the target mRNA. Bioinformatic approaches suggest that miRNAs represent 1% of all human genes and yet over a third of the transcriptome is regulated by these miRNAs[260]. Individual miRNAs can regulate hundreds of genes directly and thousands indirectly[261,262]. After the initial discovery of Lin-4 in *C. elegans*[263] in 1993, which was then believed to be an idiosyncrasy, miRNAs gained tremendous interest when a second temporally expressed miRNA let-7 was discovered in year 2000[264]. Rapidly growing interest led to the discovery of an entirely new class of small RNAs with potential regulatory roles in *C. elegans*[265-267] and in plants[268,269]. In 2002, the Croce lab showed that miRNAs were involved in cancer[270] indicating the ever growing importance of miRNA in biology. In 2003, *in vivo* experiments revealed that miRNAs could be targeted *in vivo*[271]. This finding indicated that miRNAs could be novel therapeutic targets in the near future and led to an enormous effort to understand miRNAs.

1.4.1.2.microRNA biogenesis

miRNAs are located between genes in the genome for the most part but 25% are intronic miRNAs[272]. miRNAs are often located within large 5' biased introns[273] of short genes suggesting an evolutionary advantage due to the interaction with the pre-mRNA splicing machinery[274] and the regulatory signals from the 5'UTR of the host gene[273]. Recent reports also identified nucleolar miRNAs with a potential role in late stage ribosome assembly[275,276] or miRNA interfering with other miRNA function[277]. As shown in Fig.1.13, in humans and animals, miRNA gene transcription is typically performed by RNA polymerase II (pol II) generating primary transcripts (pri-miRNAs) that are further processed into hairpin-structured miRNA precursors (pre-miRNAs) in the nucleus by RNase III Drosha and its partner Pasha/DGCR8. Pre-miRNA have 5' phosphate and a 2-nt 3' overhang and are exported out of the nucleus to the cytoplasm by Exportin5[278] where they are converted to 19-22-nt mature miRNAs by Dicer. One strand of the mature miRNA duplex is incorporated into the Argonaut 2 complex (Ago2). Ago2 has a 5'-nucleotide base-specific bias for the recognition of the miRNA guide strand, which contributes to selecting one of the two strands of the duplex[279]. miRNA-guided Argonauts form the RNA-induced silencing complex (RISC) that mediates mRNA silencing or inhibition of translation. The miRNA pathway and machinery is highly conserved and is essential. Although miRNA function is initially suppressed in oocytes[280] and early embryos[281], Dicer and Drosha[282] cofactor Dgcr8[283] deficiencies cause early embryonic lethality due to embryonic stem cell differentiation defects[282-284]. Until recently, although the alteration of miRNA levels have been extensively linked with disease states, the mechanism for the stabilized or reduced miRNA expression has remained largely elusive[285]. Mature miRNAs are degraded by 5'-3'exoribonuclease XRN-2[286]. It is believed that miRNA uptake by Ago stabilizes the miRNA by shielding it from XRN2 and by this process miRNAs with more targets will be relatively stabilized compared to the passenger strand miRNA with fewer or no targets. This model is referred to as the "use it or lose it" model[286].

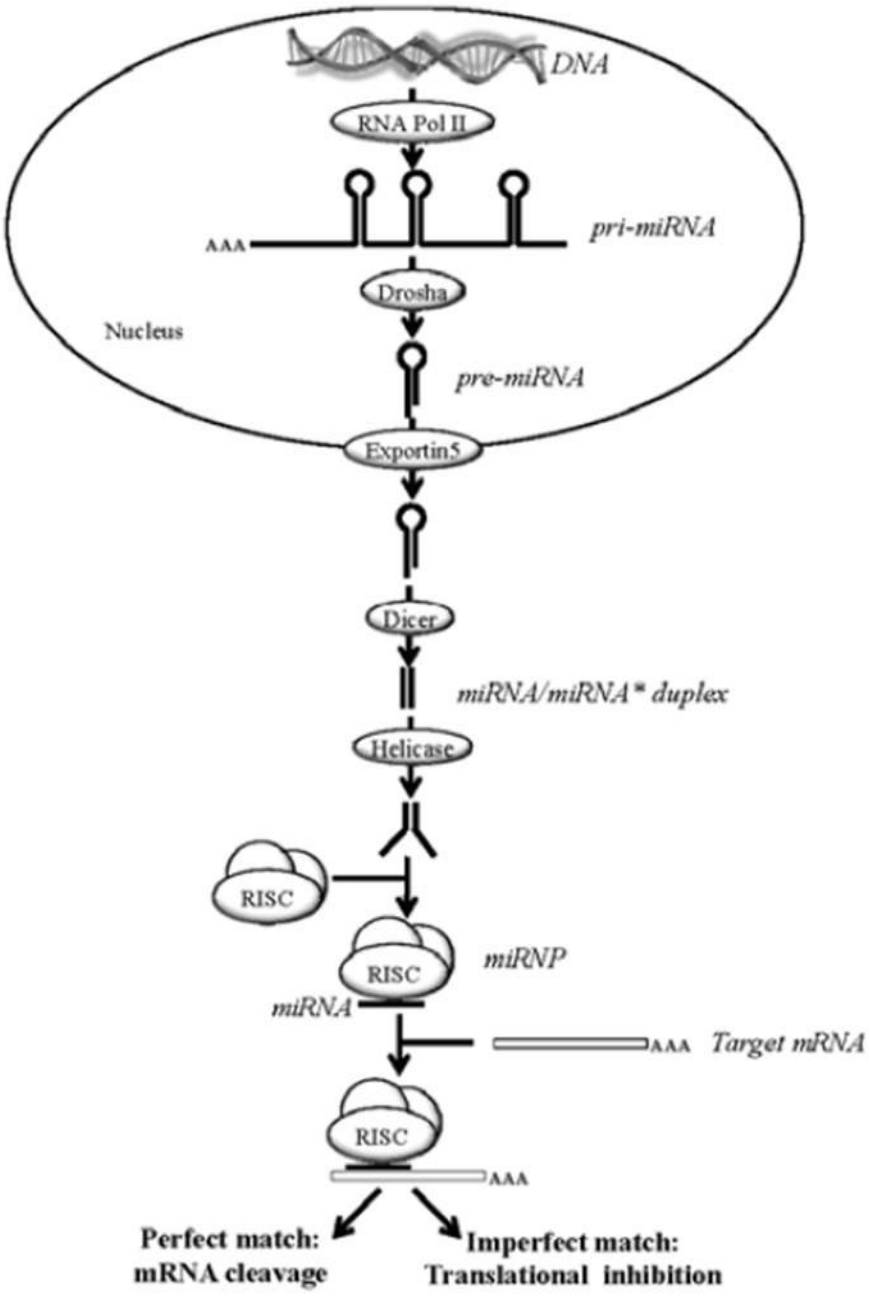


Fig.1.13 MicroRNA biogenesis:

MicroRNAs are often initially transcribed as polycistronic primary miRNA (pri-miRNA) by RNA polymerase type II. Pri-miRNAs are processed by dsRNA-specicific enzyme Drosha which isolates hairpin structures called precursor miRNAs (pre-miRNA). In some cases intronic miRNAs (mirtrons) resulting from slicing of pre-mRNAs are directly expressed as hairpins structures thereby short-circuiting the microprocessing by RNA pol II and Drosha. Pre-mIRNAs are then exported to the cytoplasm by Exportin5 where the loops are sliced by Dicer forming miRNA/miRNA* duplexes. Helicases isolate each strand before incorporation into the RNA induced silencing complex (RISC) forming the microribonucleoproteins (miRNPs). miRNPs then scan the transcriptome and bing to their cognate targets. Perfect match leads to mRNA cleavage whereas imperfect match inhibits translation initiation.

1.4.1.3.microRNA function

miRNAs are master regulators in the genome and have been shown to regulate hundreds of genes. Transfection of cells with brain-specific miR-124 caused a shift of mRNA profile towards that of the brain and similarly a transfection with muscle-specific miR-1 caused a shift towards that of muscle[287]. miRNAs are powerful master regulators of the transcriptome[287]. Overexpressing a single miRNA affects the transcriptome on a global scale by affecting thousands of mRNAs in the cell[261,288]. miRNAs regulate gene expression by guiding the RISC complex to specific binding sites of the 3'untranslated region [289](3'UTR) or coding region[289,290] or sometimes 5' UTR[291] of the target mRNA. Computational analysis has been developed to predict miRNA target mRNAs primarily based on a conserved seed paring, on local sequence and on duplex thermodynamic stability. Based on such studies of 3'UTRs, miRNAs are estimated to have hundreds of potential target mRNAs. Overall, 20-30% of human transcripts have conserved miRNA binding sites in their 3'UTRs. Identifying miRNA targets has been a challenge for the scientific community and several efforts have been made in developing public algorithms such as Miranda[292], PicTar[293], TargetScan[294,295]. Such algorithms determine possible miRNA target genes based on sequence complementarity and conservation, secondary structures and sequence accessibility. Although the false positive rates for individual target sites started at 35% for Miranda[292], improvements of such algorithms have decreased the false positive rate as the scientific community gained further knowledge of the mRNA recognition process. On average, the estimated false positive rate of the different algorithms is about 40%[296]. The inefficiency of current algorithms to effectively identify miRNA relevant target genes has been a major limiting factor in this ever growing field. miRNA binding to the target mRNA typically leads to decreased protein output either via mRNA destabilization or via translational repression. Such miRNA-mediated translational inhibition is more potent at the endoplasmic reticulum[262]. miRNA-guided RISC scans 3'UTRs of mRNAs. The nucleotides 2-7 of the miRNA are referred to as the seed and are believed to confer most of the function to the miRNA. An estimated 60% of miRNA targets can be attributed to the presence of a seed motif on the 3'UTR [261] but still a large proportion of targets remain unaccounted for. Some identified miRNA targets lack a miRNA seed[297]. The precise mechanism and proportion of miRNA-

mediated mRNA silencing or translational inhibition remain poorly understood. The first *in vitro* evidence of miRNA function was the anti-apoptotic factor miR-21 in human glioblastoma cells[298]. The aberrant increased expression of the miRNA, maintained cells in an inappropriate primitive and proliferative state. Blocking miR-21 with 2'O-methyl-modified oligonucleotides and locked nucleic acids (LNAs) resulted in undetectable levels of miR-21 and increased apoptosis[298].

1.4.2. microRNAs and cancer

By controlling and regulating the expression of one third of the genes present in the genome, it became apparent that miRNAs play a central and critical role in the pathogenesis of human diseases, including cancer (**Table 1.5**). Half of the miRNAs are located on fragile sites of chromosomes suggesting that they could play major roles in cancer[299]. Indeed, cancer-specific chromosomal rearrangement studies have shown that half of breakpoints coincide with fragile chromosomal sites[300]. Moreover, about half of the miRNA encoding genes are themselves located in chromosomal regions that are being altered during tumorigenesis[301]. Furthermore, characteristic miRNA signatures have now been profiled for many different types of tumors[302]. Early studies showed that miRNA expression profiles could discriminate between normal and cancer breast tissues more efficiently than mRNA expression profiles[303]. An elegant study from the Croce lab showed that a small miRNA signature comprised of 13 miRNA was associated with prognostic factors and disease progression indicating that miRNAs could serve therapeutic markers[304]. Since then, numerous miRNA signatures have been characterized in cancers. For instance, miR-21, miR-96 and miR-182 levels have individually been shown to correlate with poor disease-free survival in breast cancer[305], prostate cancer[306] and glioma[307] respectively. In a study on prostate cancer, miR-205 and miR-183 together could distinguish cancer from normal tissue with 84% accuracy[306]. Studies identified overexpression of miRNA (miR-200) in 70% of the pancreatic cancer samples tested[308]. While some miRNAs exert their effects as classical oncogenes or tumor suppressors[309], others act in the advanced stages of the disease by promoting cancer progression and tumor metastasis[310-313].

The tumor suppressor p53, which is mutated in 50% of human cancers directly regulates expression of several miRNAs[314,315]. Genes encoding miR-34 family are direct transcriptional targets of p53[315] and reflect p53 cellular levels. p53-regulated miR-34 suppresses proliferation and anchorage-independent growth revealing a new piece of the puzzle for p53 tumor suppressive effects[316]. Loss of miR-34 among others in colorectal[317,318] and oral squamous cell[319] carcinoma has been shown to be due to epigenetic silencing by CpG island methylation and histone modifications. miRNA levels are controlled both at the gene transcription level and at the epigenetic gene silencing level. Similarly, the oncoprotein Myc, which is typically activated in cancer cells was shown to contribute to a widespread miRNA repression[320]. Although selected miRNAs are up-regulated in cancer, global miRNA levels decrease in tumors. Among other, miR-30 and miR-26 were shown to be down-regulated by Myc[320]. Myc was shown to up-regulate the transcription of miR-17-92 cluster on chromosome 13 as well as E2F1 transcription. Interestingly, miR-17-92 regulates translation of E2F1 mRNA allowing a tightly controlled proliferative signal[321]. On the other hand, miR-31[322] and miR-200[323] play a positive role in the induction of breast cancer metastasis (Fig.1.14). Motility-relevant miR-31 targets include ITGA5, RDX, and RhoA[322,324] whose concurrent down-regulation phenocopy miR-31-mediated effects on metastasis[322]. miR-200c suppresses tumorigenicity and clonigenicity of breast cancer-initiating cells[323].

miRNA	Target	Context	Pathway	TGFβ regulation
TUMOR SUPPRESSOR MIRNAS				
miR-206	ESR1 (Adams *et al.*, 2007)	Breast cancer	ER signaling	BMP-2 in myoblast (Sato *et al.*, 2009)
miR-17-5p	AIB1, CCND1, E2F1 (Hossain *et al.*, 2006)	Breast cancer	Proliferation	
miR-125a,b	HER2, HER3 (Scott *et al.*, 2007)	Breast cancer	Anchorage-dependent growth	Liver (Huang *et al.*, 2008b)
miR-200	BMI1, ZEB1, ZEB2 (Gramantieri *et al.*, 2008; Gregory *et al.*, 2008)	Breast cancer	TGF-β signaling	Liver (Huang *et al.*, 2008b), breast (Kong *et al.*, 2009)
let-7	H-RAS, HMGA2, LIN28, PEBP1 (Yu *et al.*, 2007)	Breast cancer	Proliferation, differentiation	Breast (Kong *et al.*, 2009)
miR-34a	CCND1, CDK6, E2F3, MYC (Yu *et al.*, 2007)	Breast cancer	DNA damage, proliferation	
miR-31	FZD3, ITGA5, M-RIP, MMP16, RDX, RHOA (Valastyan *et al.*, 2009)	Breast cancer	Metastasis	Breast (Kong *et al.*, 2009)
miR-181	(Cheng *et al.*, 2005),	Lung cancer	Cell growth	Liver (Huang *et al.*, 2008b)
mir-335	SOX4, TNC (Tavazoie *et al.*, 2008)	Breast cancer	Metastasis	
miR-23-24-27	FADD (Cheng *et al.*, 2005; Chhabra *et al.*, 2009)	Lung cancer Kidney	Cell growth Apoptosis	Liver (Huang *et al.*, 2008b)
ONCOGENIC MIRNAS				
miR-21	BCL-2, TMP1, PDCD4, PTEN, MASPIN (Chan *et al.*, 2005; Cheng *et al.*, 2005)	Breast cancer, lung cancer	Apoptosis Proliferation	Liver (Huang *et al.*, 2008b)
miR-155	RHOA (Kong *et al.*, 2008)	Breast cancer	TGF-β signaling	Breast (Kong *et al.*, 2008)
miR-10b	HOXD10 (Ma *et al.*, 2010)	Breast cancer	Metastasis	
miR-373/520c	CD44 (Huang *et al.*, 2008a)	Breast cancer	Metastasis	
miR-181	TIMP3 (Wang *et al.*, 2010) BCL-2 (Chen *et al.*, 2010)	Liver cancer Glioma	Invasion Apoptosis	Liver (Huang *et al.*, 2008b)
miR-23-24-27	SMAD3 (Rogler *et al.*, 2009)	Liver cancer	Apoptosis	Liver (Huang *et al.*, 2008b)
miR-25	BIM (Li *et al.*, 2009)	Liver cancer	Apoptosis	Liver (Huang *et al.*, 2008b)

Table 1.5: microRNAs in cancer (From Humbert *et al.* 2010[14]) Numerous miRNA have been associated with cancers during the early years of miRNA research. Many studies focused on breast cancer possibly explaining the abundance of breast cancer-related miRNA.

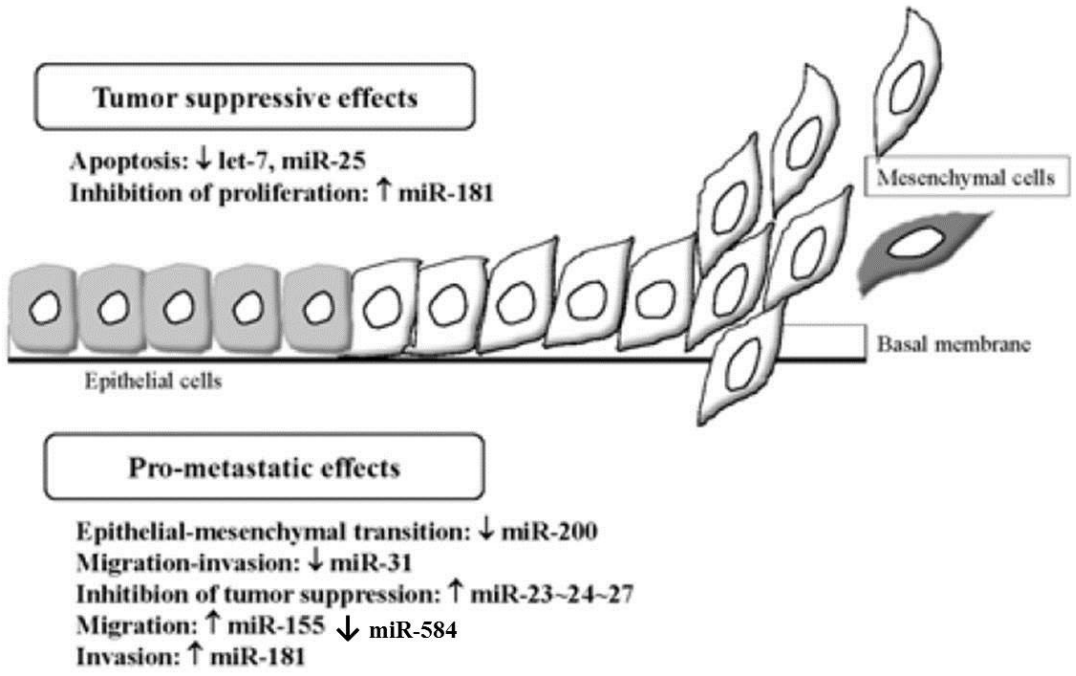


Fig.1.14: TGFβ-regulated miRNA in cancer (From Humbert *et al.* 2010[14])

In early carcinoma tumor suppressor miRNAs are up-regulated by TGFβ whereas oncogenic miRNAs are down-regulated, mediating the TGFβ tumor suppressive functions. In advanced stages miRNA regulation causes inhibition of the tumor suppression, as well as induction of epithelial-to-mesenchymal transition, migration and invasion, leading to metastasis.

1.4.2.1.Targeting miRNA in human cancer

The therapeutic potential of tethering miRNA levels is enormous. Indeed, blocking expression of specific miRNAs linked to the development of human diseases and conversely, mimicking those miRNAs involved in the prevention of diseases, could prove extremely useful. However, detailed information on the mechanism of action of specific miRNA will be a pre-requisite as blocking/antagonizing or mimicking boosting one miRNA may also impact on expression of "unwanted" genes, thus leading to off-target effects. The development of new therapeutic strategies using miRNA inhibitors and miRNA mimicking molecules is already underway for diverse human diseases, such as cardiovascular disease, neurological disorders, viral infection and of course cancer. The existing drug candidates are all nucleic acid-based therapeutics, mostly using Locked

Nucleic Acid (LNA) technology, which produces second generation antisense oligonucleotide drugs with very high specificity and potency. LNAs were shown to be highly effective in blocking miRNA functions *in vitro*[298,325]. LNAs are also specific and bind their target sequence with high affinity[326]. Silencing of miRNAs *in vivo* was shown to work using cholesterol-coupled antisense oligonucleotides. Cholesterol-coupled modified antisense oligonucleotides called antagomirs are able to down-regulate endogenous miRNA durably. For instance, miR-122 was undetectable up to 23 days post-injection of the inhibitor. Broad biodistribution was achieved as shown by decreased miRNA levels in all tissues except brain[271]. This is an efficient specific and long lasting approach to modulate endogenous miRNA levels *in vivo*.

Multiple studies on miRNAs linked to human cancer showed promising avenues. Silencing of miR-10b with antagomirs inhibits early stage metastasis in breast cancer without affecting primary tumor growth[325]. MiR-15, miR-16 and miR-181 have been shown to exert pro-apoptotic functions by targeting Bcl-2[327], thus miRNA-mimicking drugs could be developed to induce apoptosis in therapy. MiR-214 was shown to induce cisplatin resistance by targeting PTEN[328] so a cisplatin therapy combined with antisense treatments against miR-214 may increase the effectiveness of the strategy. Let-7 sensitizes cells to radiotherapy by targeting K-Ras[329] so treating patients with Let-7 mimicking drugs might render radiotherapy more efficient thereby decreasing the risk of relapse. Increasing miR-200 and decreasing miR-21 in pancreatic cancer cells renders cells sensitive to gemcitabine[330] so combination therapies may help increase the effect of an equal dose of gemcitabine. MiR-21 targets LRRFIP/TRIP resulting in the activation of the NFκB pathway[331]. Decreasing miR-21 increases sensitivity of glioma cells to 5'FU *in vitro*[332] and inhibits EGFR independently of PTEN status[333]. Increasing miR-200 and let-7 in gemcitabine-resistant cells leads to mesenchymal-to-epithelial transition[334]. Targeting the ZEB1/SIP1-miR-200 loop with miR-200 mimicking drugs is a promising approach as it would affect EMT-driven cell motility, invasiveness and drug resistance[335]. MiR-221 and miR-222 have been shown to target ERα and to confer tamoxifen resistance in MCF7 breast cancer cell line models *in vitro* indicating that targeting these endogenous miRNA could be a beneficial strategy to combine with chemotherapy[336].

1.4.2.2.miRNA and TGFβ in cancer therapy

Considering the prominent role of TGFβ in regulating tumor formation and tumor promotion, targeting specific TGFβ-regulated miRNAs may provide critical therapeutic opportunities in the near future by mimicking miRNAs that mediate the TGFβ tumor suppressive effect or by antagonizing the effects of those miRNAs that relay the TGFβ-induced tumor metastasis (**Fig. 5**). Several studies have linked TGFβ-regulated miRNA to TGFβ-mediated tumor suppression or tumor promotion (Table 4). On the growth inhibition side, the miR-106b-25 cluster targets the cyclin dependent kinase inhibitor p21 and the pro-apoptotic BCL-2 Interacting Mediator of cell death (BIM), impairing TGFβ-mediated cell cycle arrest and apoptosis respectively[337]. Similarly, overexpression of miR-25 prevents TGFβ-mediated apoptosis[337] while levels of miR-106b determine whether TGFβ can have tumor-suppressive effects. Indeed, miR-106b targets p21[338]. The miR-23a~27a~24-2 cluster is up-regulated by TGFβ in liver cancer and inhibits TGFβ-induced tumor suppression[339] by its ability to target SMAD3, which mediates tumor suppressive effects of TGFβ. Mir-181 is up-regulated by TGFβ in liver cells, which promotes carcinogenesis through the targeting of tissue inhibitor of metalloproteinase 3 (TIMP3)[162], which causes an increase in metalloproteinase activity leading to tissue plasticity.

Several miRNAs have now been shown to be involved in TGFβ-mediated tumor metastasis. For instance, TGFβ represses expression of miR-200 leading to increased levels of the miR-200 targets, among, which the transcription factor ZEB2/SIP1[154]. As ZEB2/SIP1 acts as the main repressor for E-Cadherin expression, the TGFβ-mediated decrease in miR-200 leads to decreased E-cadherin level and EMT in breast[340], pancreatic[341] and colorectal cancer[342]. In turn ZEB2/SIP1 targets TGFβ and miR-200 transcription in a feed forward loop, which stabilizes EMT[342]. MiR-155 is regulated by TGFβ and targets RhoA contributing to epithelial plasticity[343]. Thus, modulating the expression levels of the TGFβ-regulated miRNA could provide us with efficient

therapeutic means by either mimicking the TGFβ tumor suppressive effects or specifically blocking the TGFβ pro-metastatic signaling cascade.

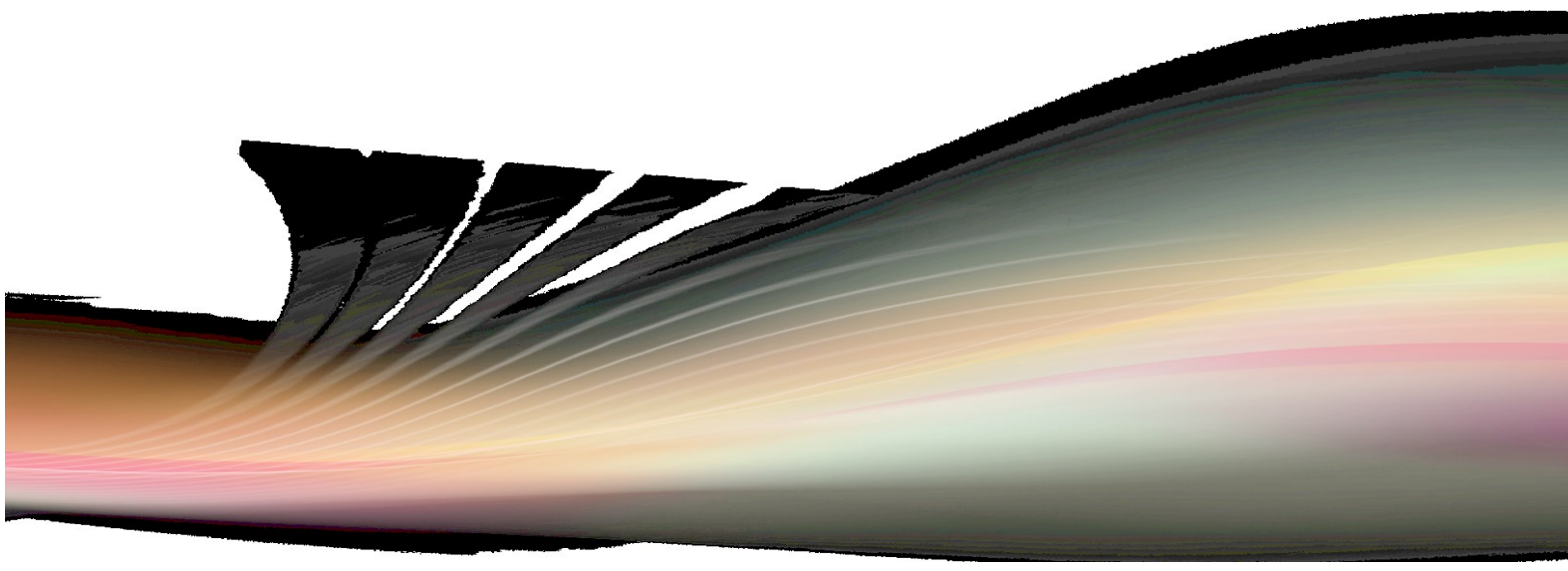
1.5.Rationale

TGFβ is a key player in tumor formation and cancer progression. Elucidation of the intracellular signaling pathways and molecular mechanisms that trigger the TGFβ tumorigenic effects have led academic and pharmaceutical laboratories to develop numerous novel anti-cancer therapies, mainly based on the use of TGFβ antagonists. Many efforts have been made to develop these new therapeutic tools and some have given promising results. Although the available tools show relative efficacy in several types of cancer, they also encounter some limitations. Indeed, because of the dual role of TGFβ in cancer, there is a need to specifically block the tumor promoter arm of the TGFβ pathway without inhibiting its tumor suppressive effects. Manipulating different miRNA levels could be a promising approach to specifically modulate the tumor-suppressive arm of the TGFβ signaling pathway while at the same time inhibiting the pro-metastatic arm of the cascade. As TNBC currently have little to no effective treatments, we investigated the possibility of using synthetic nucleic acids to target cancer-deregulated miRNA. We assessed miRNA deregulations in TNBC and attempted to address these numerous deregulations by introducing synthetic miRNA mimics or inhibitors. In this context, the main goal of my thesis was to investigate the biological role of miRNA in the downstream signaling cascade of the TGFβ superfamily. The following work sheds light on the possibility of using chemically modified antisense oligonucleotides targeting endogenous miRNA to modulate cellular responses to pro-metastatic signals. Such an approach constitutes a new highly valuable strategy in cancer therapy and would potentially allow targeting pro-metastatic signals without affecting tumor suppressive pathways thereby eliminating the problems associated with TGFβ inhibitors.

Chapter 2

Activin and TGFβ regulate expression of microRNA-181 family to promote cell migration and invasion in breast cancer cells

Published in Cellular Signalling (2013), 25(7):1556-1566



Context of the study: As described in the introductory Chapter 1, in breast cancer TGFβ has been extensively studied but to date no effective treatments blocking the tumor-promoting arm of TGFβ signaling while maintaining the tumor suppressor arm have been developed. Indeed, TGFβ both promotes late stage breast cancer and inhibit proliferation of normal breast cells and early stage breast cancers. In this study, I worked on elucidating the effect of microRNA modulation in metastatic triple-negative breast cancer cells on TGFβ prometastatic response. I identified miR-181 as a mediator of TGFβ prometastatic effect in highly metastatic triple-negative breast cancer cells.

2.1. Abstract

MicroRNA-181 (miR-181) is a multifaceted miRNA that has been implicated in many cellular processes such as cell fate determination and cellular invasion. While miR-181 is often overexpressed in human tumors, a direct role for this miRNA in breast cancer progression has not yet been characterized. In this study, we found this miRNA to be regulated by both activin and TGFβ. While we found no effect of miR-181 modulation on activin/TGFβ-mediated tumor suppression, our data clearly indicate that miR-181 plays a critical and prominent role downstream of two growth factors, in mediating their pro-migratory and pro-invasive effects in breast cancer cells. Thus, our findings define a novel role for miR-181 downstream of activin/TGFβ in regulating their tumor promoting functions. Having defined miR-181 as a critical regulator of tumor progression *in vitro*, our results thus highlight miR-181 as an important potential therapeutic target in breast cancer.

2.2. Introduction

The TGFβ ligands are multitasking cytokines that play important roles in embryonic development, cell proliferation, motility, invasion and apoptosis, extracellular matrix production and modulation of immune function[14,17,108,109,344]. TGFβ, the founding member of this family, and its receptors are expressed everywhere in the body and deregulation of the TGFβ signaling pathways have been implicated in multiple human diseases[20]. TGFβ

plays a dual role in cancer: it limits proliferation in epithelial cells and early-stage cancer cells, whereas in late stage cancer, it accelerates cancer progression and metastasis[14,54,345-347]. In the cancer niche, TGFβ can be produced and secreted into the extracellular environment by both cancer cells and host cells, such as lymphocytes, macrophages and dendritic cells. In cancer patients, high levels of TGFβ at tumor sites correlate with high histological grade, risk of metastasis, poor response to chemotherapy, and poor patient prognosis[346]. TGFβ interacts with and signals through two transmembrane serine/threonine kinase receptors (TβRI/ALK5 and TβRII), which then activate the Smad family of transcription factors (Smad2 and 3)[14,17,348].

Another member of the family, activin was initially isolated from gonadal fluid[349,350] based on its ability to induce FSHβ secretion and regulate the anterior pituitary function[16,351,352]. Activin was later shown to regulate cell growth, apoptosis and differentiation in a variety of tissues, including breast cancer[15,42,108,353,354]. Similar to TGFβ, activin initiates its signaling through ligand binding to the activin type II receptors at the cell surface, leading to the recruitment and phosphorylation of the type I receptor (ALK4)[354,355]. The activated ALK4 in turn phosphorylates the two intracellular Smad2 and Smad3, the main activin/TGFβ downstream mediators and further lead to their association with the common partner Smad4[32].

Activin and TGFβ signaling is not limited to the canonical Smad pathway, as they have also been reported to transduce their signal through non-Smad signaling pathways[14,32,42,44,54,354-356]. While the role of TGFβ in mammary gland and breast cancer has been well characterized, the role and function of activin in this tissue remains largely unknown. In breast tissue, activin and its receptors are expressed during lactation [357] and activin was suggested to participate in mammary epithelium development[358]. In breast cancer, activin can act as a tumor suppressor by inducing cell growth arrest[42,359], apoptosis[360] and by inhibiting telomerase activity[43,361]. However, even though circulating levels of activin have been correlated to bone metastasis in breast cancer[362] and that inhibiting activin was shown to prevent cancer-induced bone destruction *in vivo*[363], a direct role for activin in promoting breast cancer cell invasion and metastasis has yet to be demonstrated.

MicroRNAs (miRNAs) are a novel class of small non-coding RNAs, which have eluded researchers for decades stealthily regulating many of the major biological processes in eukaryotic cells by regulating their target genes post transcriptionally. In the past decade, our understanding of miRNA has grown tremendously from an observed oddity in worms[263] to the establishment of a fully recognized new class of regulatory molecules. They are a novel class of small (19-25nt) non-coding RNAs, which play important roles in development. Bioinformatics approaches suggest that miRNAs represent 1% all human genes and yet over a third of the transcriptome is regulated by these miRNA[260] . It became apparent that miRNA play and central and critical role in human diseases, including cancer. Half of the known miRNAs are located on fragile sites of the chromosomes suggesting that they could play major roles in cancer[299]. Cancer-specific chromosomal rearrangement studies have shown that half of the breakpoints coincide with fragile chromosomal sites[300]. Half of the miRNA-encoding genes are located in chromosomal regions that are altered during tumorigenesis[301]. Both TGFβ and activin have been shown to regulate miRNAs *in vitro*[311,364] although very little work has been done on the latter regulation. The role of miRNAs in the progression of breast cancer (BC) is emerging only recently. Several miRNA have been implicated in several steps of breast cancer progression (reviewed in[14]). For instance, miR-31 has been shown to target several genes involved in breast cancer metastasis [365]and miR-200 has been shown to target ZEB2, a transcription factor involved in EMT[366]. We also recently found TGFβ-mediated down regulation of miR-584 to be critical for breast cancer cell actin skeleton reorganization and cell motility[367].

In this study, we identified miR-181 as a potent regulator of activin and TGFβ signaling in human breast cancer. We found miR-181 to be a Smad2/3-dependent downstream target of TGFβ/activin signaling. Furthermore, our data demonstrate that activin, like TGFβ, acts as a potent inducer of cell migration and cell invasion in human breast cancer cells, thus highlighting a novel function for this growth factor in cancer cells. Moreover, we also found miR-181 to be required for activin/TGFβ-mediated cell migration and invasion, as silencing miR-181 expression significantly antagonize these growth factors pro-invasive effects, Interestingly, while significantly blocking activin/TGFβ-induced cell

migration and invasion, modulation of miR-181 endogenous levels did not altered activin and TGFβ tumor suppressive effects in cancer cells, highlighting the therapeutic potential of small antagonists to this microRNA for breast cancer treatment.

2.3. Materials and methods

Cell culture and transfection

Human breast carcinoma MDA-MB231, SCP2, SCP3 were grown in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 2 mM L-glutamine (Hyclone) and penicillin/streptomycin (Hyclone) at 37 °C under a humidified atmosphere of 5% CO_2. MCF7, HuH7, Colo320DM and U87 cells were grown in the same conditions. WM793B cells were grown in RPMI (Hyclone) in similar conditions.

Transfections

Cells were transfected with different 100 nM miRNA mimics and inhibitors (Genepharma, Shanghai, China) or siRNA (Ambion, Life Technologies, Grand Island, NY, USA) using Lipofectamine™ 2000 reagent (Invitrogen, Grand Island, NY, USA), according to the manufacturer's protocol.

Before treatment, MDA and SCPs cells were serum starved for 24 h and stimulated with 100 pM $TGF\beta_1$ (PeproTech) in DMEM supplemented with 2 mM L-glutamine. SCP2 cells transfected with miRNA mimics or inhibitors were transfected 48 h prior to $TGF\beta_1$ treatment.

Real-Time-PCR

Total RNA was extracted using TRIzol reagents (Invitrogen). Reverse transcription of 250 ng total RNA using was carried out using on miScript reverse transcriptase (Qiagen, CA, USA) as the manufacturer's instructions in a Rotor Gene 6000 PCR detection system (Corbett, San Francisco, CA, USA). miRNA PCR thermoprofile conditions were as follows: 95 °C for 15 min, 40 cycles (94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s). All primers were purchased from Qiagen. miRNA levels were normalized to the

spliceosome U6 unit. Standard curves were not conducted so this quantification was semi-quantitative.

Cell viability assay

Following an overnight FBS deprivation, HuH7 or HaCaT cells were resuspended (1×10^5 cells.ml^{-1}) in DMEM supplemented with 2% FBS in the presence or absence of 100 pM TGFβ_1 and seeded (1×10^4 cells.ml^{-1}) in 96-well plate for 48 h. After 48 h, cells were incubated for 2 h with Thiazolyl blue tetrazolium bromide **(**MTT) (5 mg.ml^{-1} in PBS, Sigma-Aldrish, Oakville, ON, Canada). Then a 20% SDS solution in 50% Dimethyl-formamide in H_2O at pH 4.7 was added to stop the reaction and mixed to homogeneity by pipetting. The absorbance at 570 nm was measured on a plate reader (BioTeK PowerWave XS) and a 690 nm measurement was used as a reference.

Migration assay

Cells were grown in 6-well plates until confluency and serum-starved overnight. A scratch was generated in the cell monolayer in straight lines using a sterile 10 µL tip guided by a ruler. Cells were then stimulated with or without 100 pM TGFβ_1 or 500 nM activin A for 24 or 48 h. Lines were drawn under the wells so photos could later be acquired from the same area. Photographs were taken initially and at the end of the ligand stimulation under phase contrast light microscopy (Olympus IX70, ImagePro AMS) and the wound closure were quantified by Image J software (NIH freeware).

Invasion assay

Cells were serum-starved overnight and seeded 5×10^4 onto a Matrigel-coated 24-well cell culture Transwell insert (8-µm pore size; BD Biosciences). Coating was done with 30 µl of 1:3 water-diluted growth factor reduced (GFR) Matrigel (BD) into each insert of the 24-tranwell invasion plate and incubated for 2 h at 37 °C in the cell culture incubator for Matrigel gelation. Cells were seeded in starvation medium on the top chamber the precoated Transwell Insert and were stimulated or not with 100 pM TGFβ_1 for 24 h. The bottom chamber contained 10% FBS in DMEM medium, which acted as chemoattractant. After 24 h, cells from the top chamber were removed by cotton swab and invading cells

were fixed with 4% formaldehyde for 10 min and then stained with a crystal violet solution for 10 min. Images of the invading cells were photographed using an inverted microscope (Olympus IX70, ImagePro AMS) and total cell numbers were counted and quantified by Image J software (NIH freeware).

2.4. Results

2.4.1. Expression of the miR-181 family members is induced by TGF-β and activin in cancer cells of multiple origins.

miRNAs are naturally occurring small non-coding RNA molecules that play crucial functions in cells by base pairing to the 3′ untranslated region (UTR) of target mRNAs, resulting in mRNA degradation or translational inhibition. Multiple miRNAs have been implicated in human diseases[368,369]. Of particular interest, the broadly conserved miRNA family miR-181 has been implicated in various human cancers. Elevated levels of miR-181 are observed in the cancer of breast, prostate and pancreas[370]. MiR-181 has been reported to act as a tumor suppressor in glioma[371]. Interestingly, the mir-181 family has also been implicated downstream of TGFβ signaling. Indeed, miR-181b can promote hepatocarcinogenesis downstream of TGFβ by regulating metalloproteinase activities[162] and it was shown that TGFβ could up-regulate the sphere-initiating stem cell-like feature in breast cancer through miR-181[372]. A recent study in murine breast cancer model revealed that miR-181a was up-regulated by TGFβ[373]. However, the exact role of miR-181 in human cancer remains unclear. Indeed, miR-181 was reported to act as a tumor suppressor in leukemia [374]and in glioma[375] but an oncogenic miRNA or oncomir in hepatocarcinoma[162]. To start investigating the role of the miR-181 family in human cancer, we initially examined the regulation by TGFβ of the different miR-181 family members shown in **Fig.2.1.A**, miR-181a, miR-181b, miR-181c and miR-181d in different human cancer cell lines of various origins. Primer specificity for the miR-181 family members was tested and cross reactivity was important between miR-181b and miR-181d which differ by only one nucleotide (data not shown).

We used a panel of human breast cancer cell lines derived from pleural effusions (MCF7 from early breast adenocarcinoma, MDA-231 with greater tumorigenic potential, SCP2, SCP3 having strong bone and lung metastatic tropism respectively[376]). The TGFβ effect

on miR-181 expression was assessed by real-time semi-quantitative PCR in cells stimulated or not with TGFβ for 24 h. As shown in **Fig.2.1B**, TGFβ significantly up-regulated the expression of all miR-181 family members to various levels in the different breast cancer cell lines tested. MCF7 cells, however, responded mildly to TGFβ possibly due to lower levels of cell surface receptors.

Interestingly, mir-181 regulation by TGFβ does not seem to be dependent on the hormone receptor status of the cells. We next investigated whether the TGFβ effect on miR-181 expression was restricted to breast cancer and, as shown in **Fig.2.1C**, we found that TGFβ could potently induce miR-181 family members expression in a variety of human cancer cell lines, including liver cancer (HepG2, HuH7 and HLE), melanoma (WM1617, WM793B and WM278), colon carcinoma (Colo320DM), keratinocytes (HaCaT) and glioma (U87). This conserved effect of TGFβ on miR-181 expression suggested an

important role for miR-181.

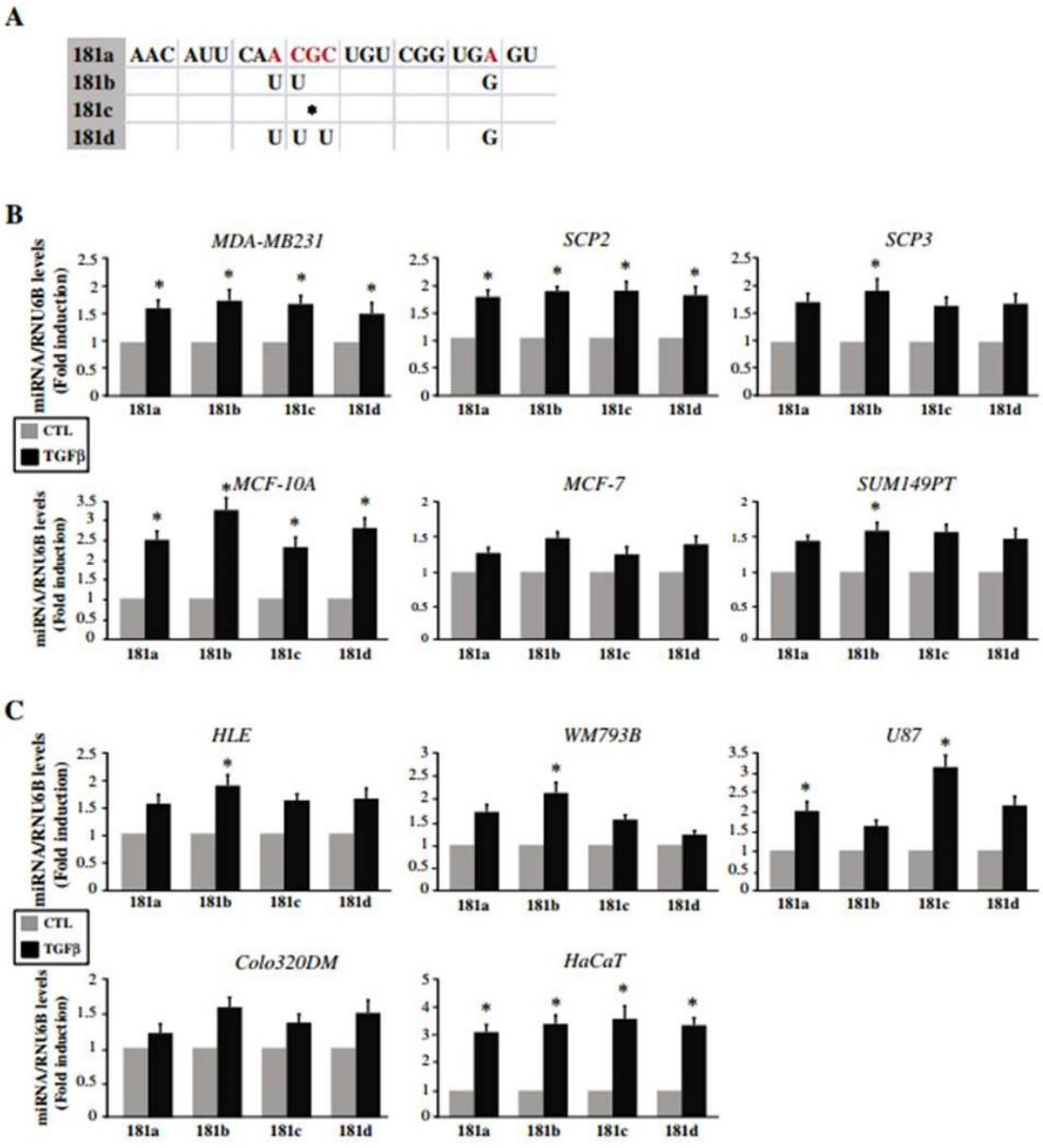


Fig.2.1. To be continued

We then further examined the regulation of miR-181 downstream of TGFβ signaling was restricted to the TGFβ ligand itself or weather it also included other members of the TGFβ superfamily. For this, we analyze the activin effect on the different miR-181 isoforms. Indeed, activin belongs to the TGFβ family and share downstream signaling pathways and molecules (Smad2, 3 and 4) with TGFβ. Interestingly, as shown in **Figs.**

2.1D and Fig.2.1E, we found that activin could significantly up-regulate miR-181a, b, c and d in a broad range of tissues.

Together, these results define the miR-181 family as a downstream target for both activin and TGFβ signaling in human cancer cells of various origins and suggest an important regulatory role for this family of microRNAs in the mediation of the activin/TGFβ responses in cancer cells.

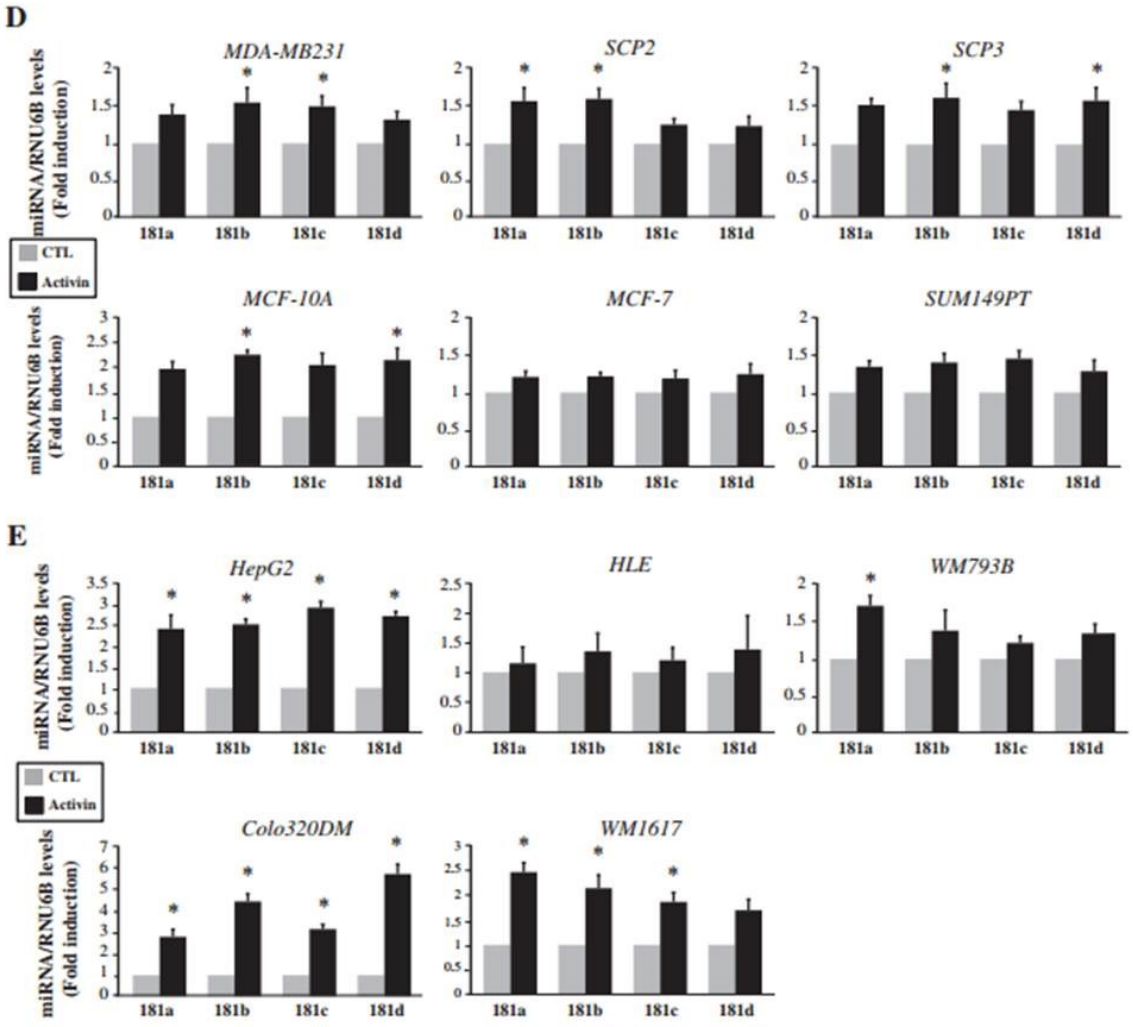


Fig. 2.1. miR-181 is a conserved target of activin/TGFβ signaling.

A, The different miR-181 family members share a common seed and differ in other miRNA regions. **B**, Breast cancer cells were serum-starved overnight and stimulated or not with 100 pM TGFβ for 24 h and miR-181 expression levels were analyzed by RT-real time PCR. **C**, Liver (HLE), melanoma (WM793B), glioma (U87), colorectal (Colo320DM) and squamous cell carcinoma (HaCaT) cell lines were treated or not with 100 pM TGFβ1 for 24 h and miR-181 expression levels were analyzed by RT-real time PCR. **D**,

Breast cancer cells were serum-starved overnight and stimulated or not with 500 nM activin A for 24 h and miR-181 expression levels were analyzed by RT-real time PCR. **E**, Liver (HepG2 and HLE), melanoma (WM1617) and colorectal (Colo320DM) cell lines were treated or not with 500 nM activin A for 24 h and miR-181 expression levels were analyzed by RT-real time PCR. Data is graphed as the geometric mean of RNU6B-normalized fold inductions of miR-181 family members in response to TGFβ for 3 independent experiments. The error bars are geometric SD. For statistical analysis the z-test was performed on the logarithmic values and ligand-treated conditions were compared to the non-treated control (* $p \leq 0.05$).

2.4.2. Activin/TGFβ-induced miR-181 expression is mediated through the canonical Smad pathway and is both Smad2 and Smad3-dependent

The Smad proteins are the main mediators of the activin and TGFβ signaling pathways[32,377]. To assess whether the activin/TGFβ-mediated induction of miR-181 expression is dependent on the Smad pathway, breast cancer cells were transfected or not with specific siRNAs against Smad2, Smad3 or a scrambled sequence as negative control and incubated in the presence or the absence of activin or TGFβ for 48 h. As shown in **Fig.2.2A** and **Fig.2.2C**, both activin and TGFβ significantly induced mir-181d expression, even though activin is slightly less potent.

Interestingly, knocking down expression of either Smad2 or Smad3 completely abolished both the activin and TGFβ effect on miR-181d. Similar patterns were observed for the other members of the miR-181 family.

We then investigated whether the activin/TGFβ non-Smad signaling pathways were also involved in the regulation of miR-181, by specifically inhibiting these pathways using chemical inhibitors (LY294002 for PI3Kinase, Rapamycin for the mTOR in the PI3K/Akt pathway, U0126 for MEK1/2 in the MAP Kinase pathway, SB203580 for the p38 and SP600125 for the JNK in the p38/JNK pathway). As a positive control we also used the activin/TGFβ type I receptor kinase inhibitor SB431542. SB431542 was characterized as a potent inhibitor of the activin, TGFβ and nodal type I receptors (ALK4, ALK5 and ALK7 respectively)[378]. As shown in **Fig.2.2B** and **Fig.2.2D**, blocking the type I activin/TGFβ kinase activity completely abolished activin and TGFβ-induced miR-181 expression. Interestingly as well, blocking the PI3Kinase pathway, using the LY294002 inhibitor also partially antagonized the activin/TGFβ effects on mir-181 expression, suggesting a role for this pathway in the regulation of the miR-181, in addition to the

Smads. Interestingly, inhibition of the MEK1/2 pathway using U0126 resulted in an increase of the TGF-β induction of mature miR-181 from 1.6-fold in vehicle-treated condition to 2.2-fold in U0126-treated condition. Taken together, our data indicate that the activin/TGFβ regulation of miR-181 expression is specifically mediated through the Smad pathway and is both Smad2 and Smad3-dependent.

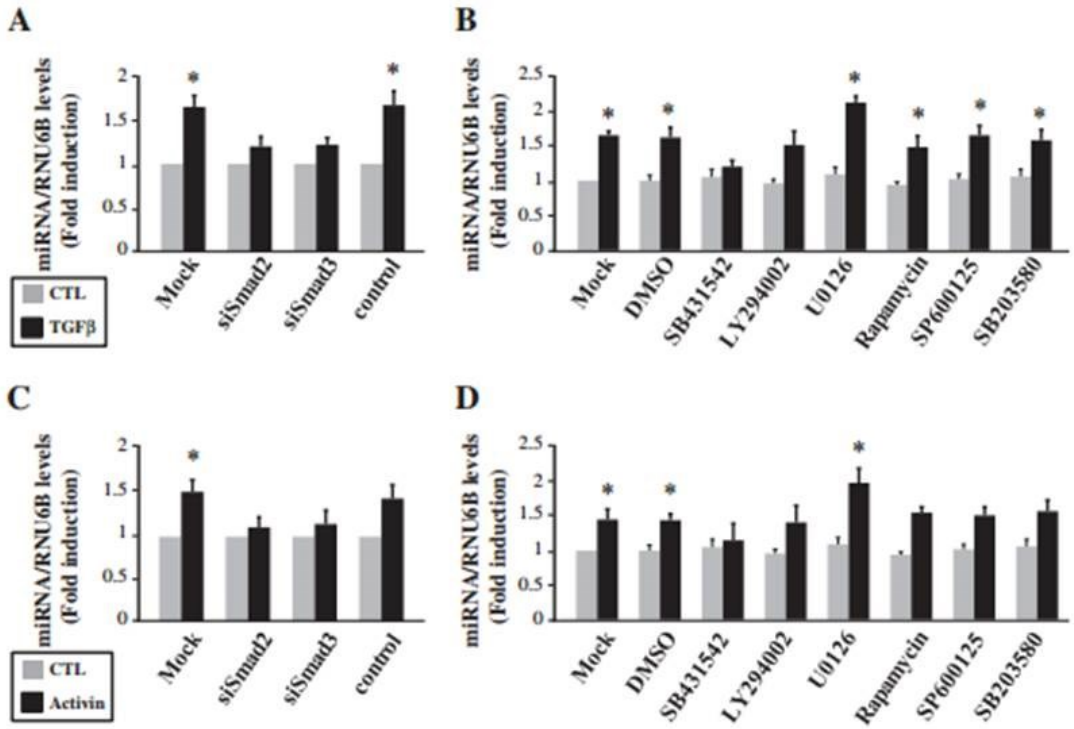


Fig.2.2: MiR-181 induction by activin/TGFβ signaling is mediated through the canonical Smad2/3 pathway.

A, SCP2 cells were transfected with Smad2, Smad3 or a control siRNA for 24 h then serum-starved overnight and treated or not with 100 pM TGFβ1. miR-181 expression levels were assessed by RT-real time PCR. **B**, SCP2 cells were serum-started overnight and pre-treated with different chemical inhibitors then treated or not with 100 pM TGFβ1. miR-181 expression levels were assessed by RT-real time PCR. DMSO was used as a vehicle control. **C**, SCP2 cells were transfected with Smad2, Smad3 or a control siRNA for 24 h then serum-starved overnight and treated or not with 500 nM activin A. miR-181 expression levels were assessed by RT-real time PCR. **D**, SCP2 cells were serum-started overnight and pre-treated with different chemical inhibitors then treated or not with 500 nM activin A. miR-181 expression levels were assessed by RT-real time PCR. DMSO was used as a vehicle control. Data is graphed as the geometric mean of

RNU6B-normalized fold inductions of miR-181 family members in response to TGFβ1 for 3 independent experiments. The error bars are geometric SD. For statistical analysis the z-test was performed on the logarithmic values and ligand-treated conditions were compared to the non-treated control (* $p \leq 0.05$).

2.4.3. Silencing and overexpression of the miR-181 using antagomirs and mimics, respectively

Several generations of chemically-modified oligonucleotides have been developed in order to deplete endogenous RNA. Initial generations of antisense oligonucleotides (ASO) were developed to target mRNA. These first generation ASO targeted and paired endogenous mRNAs leading to DNA-RNA hybrid that recruited RNAse H to cleave the mRNA[379]. In order to functionally characterize miR-181, we used chemically-modified inhibitor oligonucleotide sequences that bind to and irreversibly sequester endogenous miR-181 thereby decrease the intracellular miR-181 activity. We used double-stranded oligonucleotide mimic sequences that were processed as miRNA duplexes by cell machinery thereby elevating miR-181 activity. We were able to decrease miR-181 potently with a remaining 40% decrease 5 days post-transfection Fig.2.3A-B. We were also able to have a strong increase of miR-181 levels with an 8 to 10-fold increase 5 days post-transfection. The ability of miR-181 mimics and inhibitors to modulate miR-181 levels in a cell line-independent manner was tested in WM278 melanoma cells (data not shown).

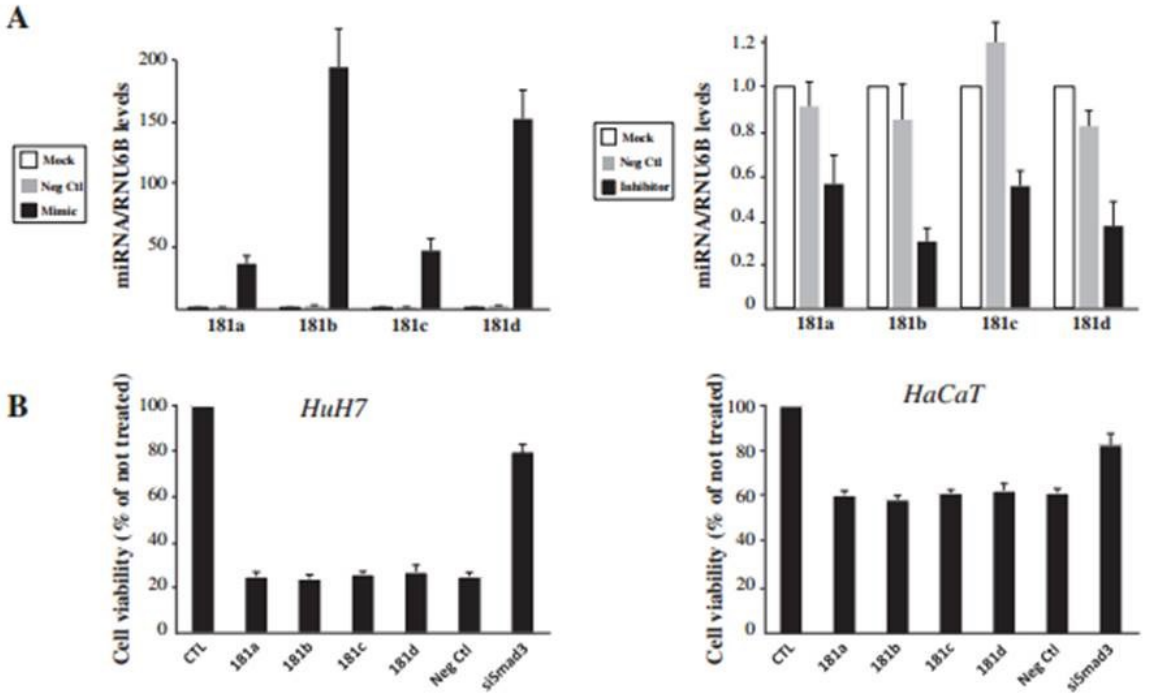


Fig.2.3: Modulation of miR-181 does not affect antiproliferative effects of TGFβ.

A, SCP2 cells were transfected with 100nM miR-181 mimics or inhibitors. Five days post-transfection miR-181 modulations were assessed by RT-real time PCR. Data is graphed as the geometric mean of RNU6B-normalized fold inductions of miR-181 family members for 3 independent experiments. The error bars are geometric standard deviations. **B**, Hepatocarcinoma (HuH7) and squamous cell carcinoma (HaCaT) cells were transfected with 100 nM miR-181 inhibitors and stimulated or not for 48 h with 100 pM TGFβ1. Cell viability was assessed by the MTT assay. Data is graphed as the arithmetic mean of cell viability (% of non-treated control) for 3 independent experiments. The error bars are SEM.

2.4.4. miR-181 modulation does not affect TGFβ anti-proliferative effects

Previous reports have indicated that miRNA modulation affected cell proliferation[380]. Moreover, both activin and TGFβ exert strong anti-proliferative effects in breast cancer[42,381] but also in other target tissues, such as hepatocarcinoma[76,146,353], pituitary tumors[351] or keratinocytes[43,382]. Thus, we examined the functional relevance of activin/TGFβ-induced miR-181 expression in mediating the activin and TGFβ anti-proliferative effects. For this, we used miRNA mimics and inhibitors to respectively increase and decrease miR-181 levels *in vitro*. Inhibition of miR-181 with individual miR-181 family members inhibitors did not affect TGFβ anti-proliferative effects as assessed by MTT (**Fig.2.3B**) in HuH7 cells and HaCaT keratinocytes. However, a partial disruption of this effect was observed following knockdown of Smad3 in all model cell lines tested indicating that Smad3 was a downstream mediator of TGFβ anti-proliferative effects. Similar results were obtained in other cell types (i.e. breast cancer) and in response to activin (data not shown). This suggests that miR-181 is not involved in mediating TGFβ anti-proliferative effects.

2.4.5. miR-181 modulation affects activin/TGFβ-induced cell migration

As miR-181 is strongly induced by activin and TGFβ signaling in breast cancer, we further assessed the functional role of miR-181 in the mediation of other effects of TGFβ in breast cancer. TGFβ exert a dual role in breast cancer, acting as a tumor suppressor in early carcinoma and as a tumor promoter in advanced malignant tumors (reviewed in[14]). Such a role for actin in the other hand has yet to be demonstrated. To further investigate the role of miR-181 downstream of activin and TGFβ in breast cancer, we first examined the effects of these two growth factors on cellular migration. For this we used a model cell line representing an aggressive, highly metastatic human breast cancer cell line, SCP2, in, which we previously found TGFβ to exert strong pro-migratory and pro-invasive effects[345]. As shown in **Fig2.4A**, TGFβ significantly promoted cell migration.

Interestingly, activin also strongly stimulated cell migration, to a level similar to what observed for TGFβ.

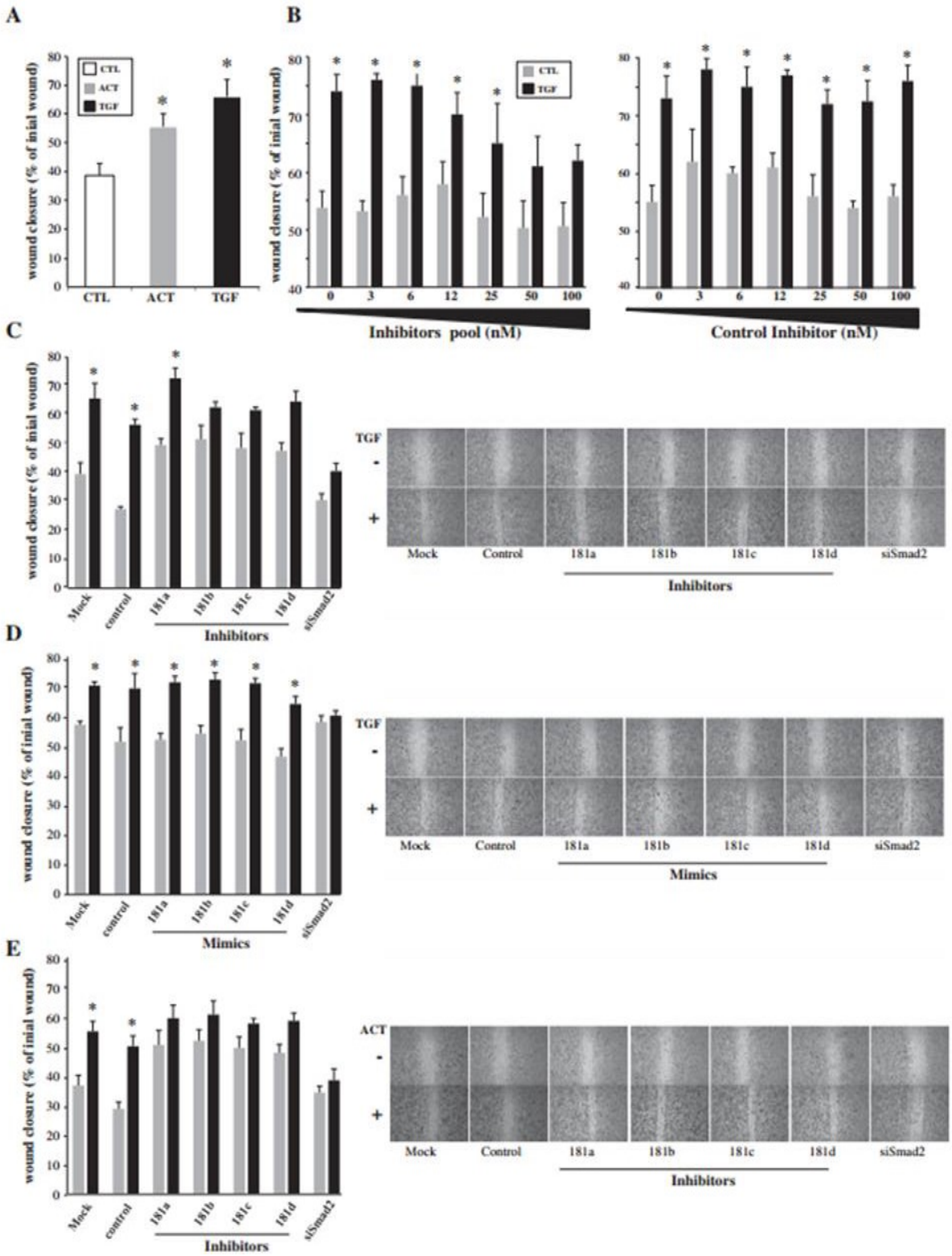


Fig.2.4:. miR-181 is required for activin/TGFβ-induced migration in breast cancer.

A, SCP2 monolayer was wounded and cells were stimulated or not with 500 nM activin A or 100 pM TGFβ1. The effect of ligand stimulation was visualized after 24 h using phase contrast microscopy. The area of the wound was measured using ImageJ software and the wound closure was graphed after normalization to the corresponding initial wound size. Data is graphed as the arithmetic mean of 4 independent experiments. The error bars are the standard error of the mean. **B**, SCP2 cells were transfected with gradually increasing concentrations of pooled miR-181 or negative control inhibitors (from 0 nM to 100 nM) and stimulated or not with 100 pM TGFβ1. The effect ligand stimulation following a gradual inhibition of miR-181 activity was visualized after 24 h using phase contrast microscopy. The area of the wound was measured using ImageJ software and the wound closure was graphed after normalization to the corresponding initial wound size. Data is graphed as the arithmetic mean of 4 independent experiments. The error bars are the standard error of the mean. **C**, SCP2 cells were transfected with 100 nM of individual miR-181 family inhibitors and stimulated or not with 100 pM TGFβ1. 100 nM siRNA targeting Smad2 was used as a control. The effect ligand stimulation following inhibition of individual miR-181 family members was visualized after 24 h using phase contrast microscopy. The area of the wound was measured using ImageJ software and the wound closure was graphed after normalization to the corresponding initial wound size. Representative photos are shown in the right panel. Data is graphed as the arithmetic mean of 4 independent experiments. The error bars are the standard error of the mean. **D**, SCP2 cells were transfected with 100 nM of individual miR-181 family mimics and stimulated or not with 100 pM TGFβ1. 100 nM siRNA targeting Smad2 was used as a control. The effect ligand stimulation following inhibition of individual miR-181 family members was visualized after 48 h using phase contrast microscopy. The area of the wound was measured using ImageJ software and the wound closure was graphed after normalization to the corresponding initial wound size. Representative photos are shown in the right panel. Data is graphed as the arithmetic mean of 3 independent experiments. The error bars are the standard error of the mean. **E**, SCP2 cells were transfected with 100 nM of individual miR-181 family inhibitors and stimulated or not with 500 nM activin A. 100 nM siRNA targeting Smad2 was used as a control. The effect ligand stimulation following inhibition of individual miR-181 family members was visualized after 24 h using phase contrast microscopy. The area of the wound was measured using ImageJ software and the wound closure was graphed after normalization to the corresponding initial wound size. Representative photos are shown in the right panel. Data is graphed as the arithmetic mean of 4 independent experiments. The error bars are the SEM (* $p \leq 0.05$).

We did not observe any synergistic effect between activin and TGFβ on cell migration (data not shown), presumably due to the fact that TGFβ and activin signal through and compete for the same Smad molecules. Our data highlight for the first time a pro-migratory role for activin in human breast cancer cell migration[383]. Activin was reported to promote migration in prostate[384] and dendritic cells[288]. There is no definitive demonstration that activin has tumor promoting potential in the mammary gland and little is known about the role of activin in oncogenic progression in breast cancer[383].

To then address the functional relevance of activin/TGFβ-induced mir-181 expression downstream of these growth factors induction of cell migration, we knocked-down miR-181 family members expression in cells stimulated or not with TGFβ and cell migration was assessed using the scratch/wound healing assay, as previously described[345]. We initially tested the effect of increasing concentrations of pooled miR-181 antagomir on

TGFβ-induced promigratory response. We observed a gradual decrease of TGFβ-induced promigratory response between 6 nM and 50 nM of pooled antagomirs. This was not paralleled in the negative control antagomir transfections **Fig.2.4B**.

We then tested the individual antagomirs of the miR-181 family members and observed that they all modulated TGFβ-induced migration but to different extents. Blocking expression of miR-181b, c, d and to a lesser extend miR-181a, slightly but significantly attenuated the TGFβ pro-migratory effect in SCP2 cells. miR-181a down-regulation was recently shown to decrease mouse 4T1 cell migration[373]. We also observed the TGFβ pro-migratory response to be Smad2-dependent. **Fig.2.4C**. The sense sequences of the same miR-181 family members had no detectable potentiating effect on the TGFβ-induced migration **Fig.2.4D**. Interestingly, our results also indicate that activin also potentiate breast cancer cell migration, even though to a lesser extent than TGFβ. **Fig.2.4E**.

To our knowledge, this is the first demonstration for such a role of activin in breast cancer. Together, our findings highlight a novel function for activin in regulating cell migration in breast cancer cells and indicate that up-regulation of miR-181 expression by these growth factors is a prerequisite step for the induction of cell migration in human breast cancer.

2.4.6. miR-181 modulation affects activin/TGFβ-induced cell invasion

Recent studies associated miRNA modulation with invasive potential such as miR-10b in hepatocarcinoma[385] and miR-183 in osteosarcoma[386]. Moreover, a recent study by Wang *et al.*[162] showed that TGFβ promoted liver cell invasion by increasing miR-181 in hepatocarcinoma, through down-regulation of tissue inhibitor of metalloproteinase 3 (TIMP3) leading to an increase activity of MMP2 and MMP9. As TGFβ is pro-invasive in breast cancer, this prompted us to investigate whether miR-181 modulation affected invasive potential in our SCP2 breast cancer model. Using Transwell/Matrigel assays, in SCP2 cells, we found TGFβ to potently induced cell invasion **Fig.2.5A**.

Quantification was performed using ImageJ. Moreover, our results also indicate that activin strongly promotes invasion of these breast cancer cells, further expanding on the new roles played by this growth factor in breast cancer. No synergistic effects between

activin and TGFβ were observed on cell invasion (data not shown). Interestingly, gradual overexpression of miR-181 led to an increased in the basal invasion rate of SCP2 cells, indicating that overexpression of miR-181 also is sufficient to mimic the activin and TGFβ effects on breast cancer cell invasion **Fig.2.5B**.

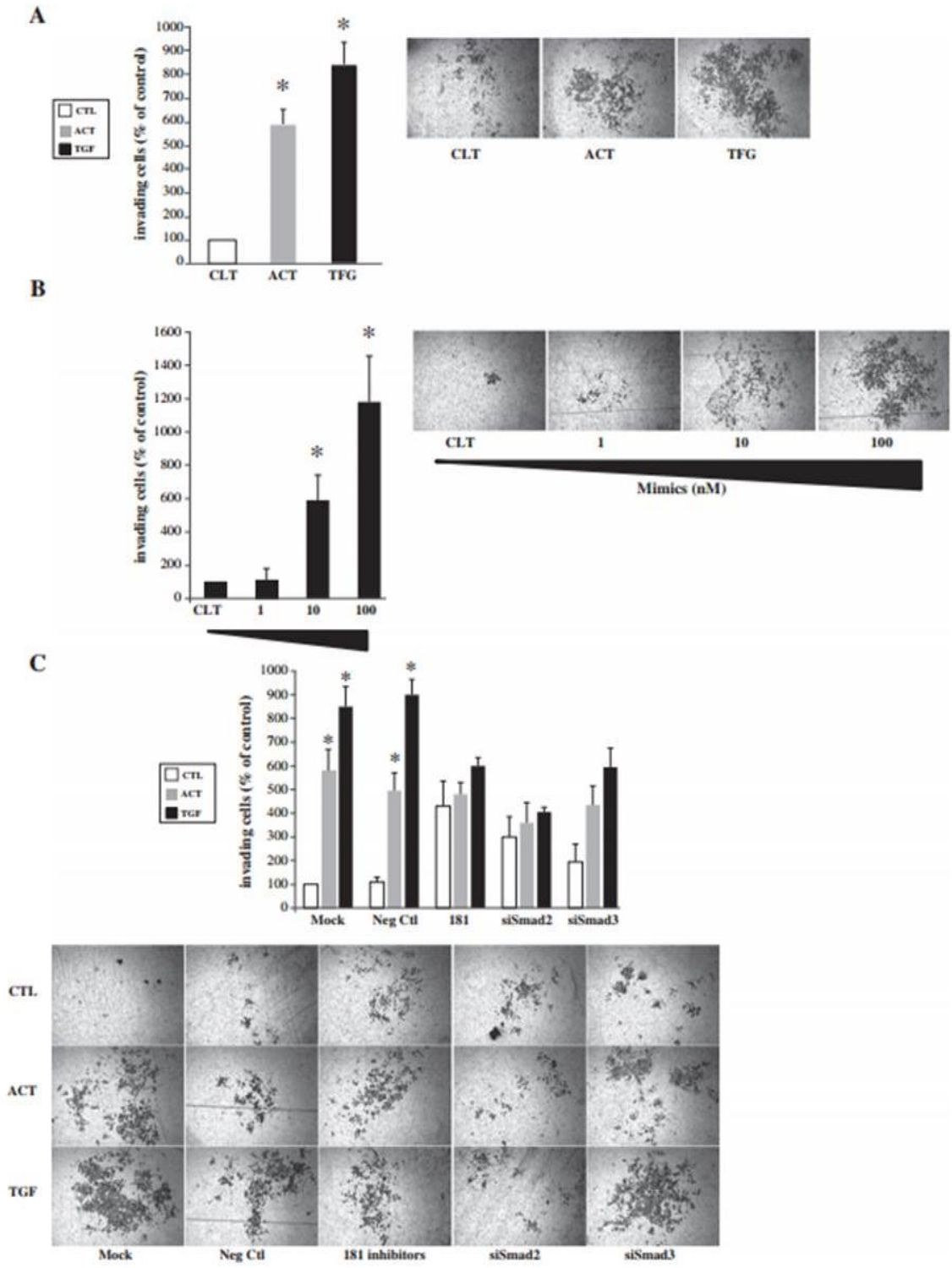


Fig.2.5: miR-181 is required in activin/TGFβ-induced invasion in breast cancer.

A, SCP2 cells were seeded in starvation medium on a Transwell cell culture insert coated with growth factor reduced Matrigel and stimulated or not with 500 nM activin A or 100 pM TGFβ1 for 48 h. The invading cells were visualized and photographed on the bottom side of the filter of the insert after crystal violet staining using phase contrast microscopy. Representative photos are shown in the right panel. Invading cells were counted using ImageJ software. Data is graphed as the arithmetic mean of 2 independent experiments. The error bars are SEM. **B**, SCP2 cells were transfected with increasing concentrations of pooled miR-181 mimics from 0 nM to 100 nM and seeded in starvation medium on a Transwell cell culture insert coated with growth factor reduced Matrigel. The invading cells were visualized and photographed after 48 h on the bottom side of the filter of the insert after crystal violet staining using phase contrast microscopy. Representative photos are shown in the right panel. Invading cells were counted using ImageJ software. Data is graphed as the arithmetic mean of 2 independent experiments. The error bars are SEM. **C**, SCP2 cells were transfected with 100 nM of pooled miR-181 inhibitors. Transfections with siRNA targeting Smad2 and Smad3 were used as controls. Transfected SCP2 cells were seeded in starvation medium on a Transwell cell culture insert coated with growth factor reduced Matrigel and stimulated or not with 500 nM activin A or 100 pM TGFβ1 for 48 h. The invading cells were then visualized and photographed on the bottom side of the filter of the insert after crystal violet staining using phase contrast microscopy. Representative photos are shown in the right panel. Invading cells were counted using ImageJ software. Data is graphed as the arithmetic mean of 2 independent experiments. The error bars are SEM (* $p \leq 0.05$)

Moreover, our results also clearly indicate that miR-181 is required for activin and TGFβ to induce cell invasion through the Matrigel, as miR-181 silencing completely blocked these growth factors effects on cell invasion. Inhibition of activin/TGFβ-mediated cell invasion was similar to what observed following gene silencing of the canonical Smad pathway, clearly indicating that miR-181 is critical to the cell invasion process **Fig.2.5C**.

It was intriguing to observe that treatment of the cells with the miR-181 inhibitor also led to an increase in basal cell invasion. This increased cell invasion is possibly due to an off-target effect of the inhibitor. MicroRNAs are known to regulate multiple intracellular targets and it is conceivable that miR-181 regulates the expression of other target genes that involved in the maintenance of cell invasion under basal conditions. Collectively, our data show that both activin and TGFβ are potent inducer of cell invasion in breast cancer cells and that their effects require the up-regulation of miR-181 gene expression.

Discussion

In this study, we describe a novel role for the microRNA miR-181 as a potent-mediator of breast cancer cell migration and invasion, Early works had shown miR-181 to be a tumor suppressor in glioblastoma[371]. The role of miR-181 in the context of breast cancer remained to be characterized. We found miR-181 gene expression to be dependent and regulated by the two growth factors, activin and TGFβ in multiple cancer cell lines of various origins. Furthermore, our data also indicate that up-regulation of miR-181 by

activin/TGFβ is required for these growth factors to mediate cell migration and cell invasion in breast cancer and suggest an important role for miR-181 in the metastatic process of this type of cancer.

Activin has been reported to inhibit cell cycle through the p38 pathway[42]. Activin induces migration in mast cells however at higher concentrations than TGFβ[387]. In colon cancer, restoration of activin signaling reveals its pro-migratory role similar to TGFβ[388]. The effect of activin on breast cancer cell migration was unclear. Al-Hajj *et al.*[389] described that the $CD44^{pos}/CD24^{pos}$ subpopulation was non tumorigenic and that $CD44^{pos}/CD24^{pos}$ subpopulation was tumorigenic in immunocompromised mice . Activin signaling has been shown to mediate the interconversation of noninvasive $CD44^{pos}/CD24^{pos}$ cells into their $CD44^{pos}/CD24^{neg}$ invasive counterparts[390]. Recent studies have shown that miRNA could modulate cancer cell migration *in vitro* in glioma[391], liver[392] and breast[393] cells.

To our knowledge, this is also the first report of a role for activin in mediating cell migration/invasion in breast cancer. Activin has been studied in the context of embryo development. Until recent studies including those in colon[394] and prostate cancer[395] few studies had focused on its role in cancer. Only one paper focused on miRNAs downstream of activin signaling[364]. We observed a conservation of the regulation of miR-181 by TGFβ through different cellular contexts suggesting an important role for miR-181 across tissues. Interestingly, we report a novel role of activin in regulating miRNA in several model cells lines. This is indeed the first report of activin-mediated miR-181 induction. This miRNA regulation provides new insight into the role of activin in different cancer models. We observed that the miR-181 induction was more potent in cells with more pronounced anti-proliferative TGFβ responses such as HuH7 and HaCaT cells where the induction was >6-fold and >3-fold respectively as opposed to ~2-fold in SCP2 cells where the prometastatic response of the cells to TGFβ is accompanied by a milder transcriptional response. Interestingly, we observed that miR-181 was induced by both activin/TGFβ, two members of the TGFβ superfamily.

We showed that the regulation of miR-181 by activin/TGFβ in this breast cancer model is dependent on the canonical Smad2/3 pathway but also requires the PI3 kinase pathway. Such a role for the PI3K pathway, downstream of TGFβ, in association with the Smads

has been documented previously. Indeed, TGFβ signals through the PI3 K pathway to regulate cell growth inhibition[49] and induction of EMT[51]. We observed that inhibiting activin/TGFβ -induced miR-181 activity with 3'O-methyl-modified antisense RNA sequences was effective and lasted up to 5-days. The inhibition of miR-181 activity resulted in impaired activin/TGFβ-induced pro-migratory responses. This is also the first report of activin promigratory effect in breast cancer. The incomplete blockade of activin/TGFβ -induced pro-migratory responses by miR-181 inhibition suggest that other miR-181-independent pathways might be mediating pro-migratory effects. Increasing miR-181 levels with miRNA mimics did not significantly increase TGFβ pro-migratory effects. These effects of miR-181 modulation however did not affect the tumor-suppressive responses of TGFβ as assessed by the anti-proliferative effects of TGFβ in any of the model cell lines tested. Together our findings highlight miR-181 knockdown as a possible strategy to inhibit pro-migratory and pro-invasive effects of activin/TGFβ signaling without interfering with the tumor suppressive arm of the pathway. Early clinical studies[113,396] concluded that increased TGFβ signaling led to increased metastasis prompting the industry to develop antagonists for the TGF-β signaling pathway. Models of TβRII knockouts showed surprisingly minimal phenotype[253,397,398] suggesting that there were compensatory mechanisms to the endogenous TGFβ growth inhibitory role. TβRII knockout however has a marked effect when combined with oncogene activation or tumor suppressor gene attenuation suggesting that the tumor suppressor arm of TGFβ is not compensated for by other signaling pathways[254]. Indeed, the study by Forrester *et al.* indicated that TβRII knockout increased lung metastases in their model of oncogene-induced mammary carcinoma. Disrupting TGFβ signaling necessarily also affected the tumor microenvironment and increases the number of myeloid immune suppressor cells, which contributes to tumor growth and vascularization[147,399]. These observations show that blocking TGFβ signaling too broadly has deleterious effects. It is in this context that miR-181 has a potential therapeutic value as it is downstream of TGFβ signaling and does not seem to be involved in the tumor suppressor arm of TGFβ signaling.

miRNA therapeutics is a growing field with potential application in liver cancer treatment[400]. MiR-181 could act as a potential therapeutic metastatic miRNA target in

breast cancer. Indeed, although numerous approaches have been undertaken over the past decade to disrupt TGFβ signaling at different levels of the signaling cascade[14], none of the methods were effective as they all resulted in some alteration of the beneficial tumor suppressor arm of the signaling cascade. This study indicates that modulating miR-181 downstream of activin/TGFβ signaling not only decreases pro-migratory and pro-invasive effects of TGFβ signaling but does not affect the tumor suppressor arm of TGFβ.

Acknowledgments

The authors are thankful to Dr. Y. Eto and Ajinomoto Co., Inc. for generously providing recombinant activin A. We thank Dr. Joan Massagué for kindly providing us the MDA, SCP2 and SCP3 cell lines, Dr. Stephen P Ethier for kindly providing us SUM149 cell line. This work was supported by grants from the Canadian Institutes for Health Research (CIHR) to JJL. JJL is the recipient of the McGill Sir William Dawson Research Chair.

Grant Support

This work was supported by a Canadian Institutes for Health Research (CIHR) grant (fund code 230670 to JJL). JJ Lebrun is the recipient of a Sir William Dawson McGill Research Chair.

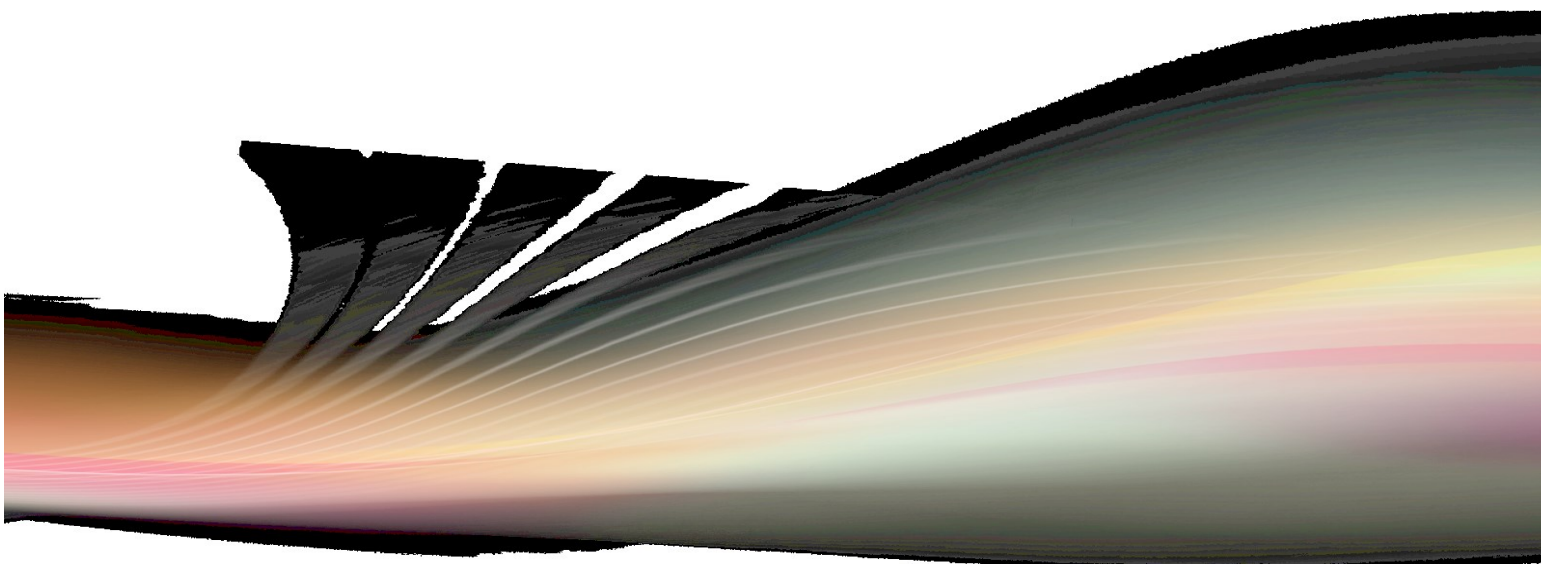
Our initial efforts have revealed that TGFβ-regulated miRNA[367,401] could potentially be of therapeutic interest in the context of metastatic breast cancer where modulating miRNA levels could decrease TGFβ pro-metastatic effects *in vitro*. We continued to investigate the role of miRNA downstream of TGFβ and sought to identify TGFβ-regulated miRNA mediating TGFβ tumor suppressive effects. Such miRNA would also be of high therapeutic interest as they could be therapeutically over-expressed to limit tumor progression. For this, we used another triple negative breast cancer model in which the tumor suppressive response to TGFβ was strong. We identified a TGFβ-regulated miRNA involved in tumor TGFβ-mediated tumor growth inhibition.

Chapter 3

TGFβ acts as a tumor suppressor in breast cancer cells by inhibiting miR-30

Manuscript to be submitted for publication

Jean-Charles Neel and Jean-Jacques Lebrun



Contribution of authors: I designed and performed the experiments which led to the data discussed in this chapter. I supervised and trained two interns who participated in the initial experiments for this work.

3.1. Abstract

MicroRNA-30 (miR-30) is a multifaceted non-coding RNA involved in many biological processes such as anoikis, radiotherapy resistance and angiogenesis. Although decreased levels of miR-30 are often observed in tumors, the direct involvement of miR-30 in breast cancer progression has not yet been clearly characterized. We showed this miRNA to be inhibited by TGFβ and this regulation to be necessary for TGFβ-induced tumor suppression. Our data clearly reveals miR-30 to play a prominent role in the TGFβ tumor suppressive response in breast cancer cells. We identified novel TGFβ targets genes downstream of miR-30 implicated in TGFβ tumor-suppressive effects. Our findings position miR-30 downstream of TGFβ signaling and as a key element of the tumor suppressive functions of TGFβ. This study highlights miR-30 as a potential target in breast cancer therapy.

3.2. Introduction

TGFβ is a pluripotent cytokine that play important roles in embryonic development, cell proliferation, apoptosis, immortalization and cancer progression[14,17,43,108,109,344]. TGFβ, the founding member of this family, and its receptors are ubiquitously expressed throughout the body and deregulation of the TGFβ signaling pathways have been implicated in numerous diseases[20]. TGFβ interacts with and signals through two transmembrane serine/threonine kinase receptors (TβRI/ALK5 and TβRII), which then activate the Smad family of transcription factors (Smad2 and 3)[14,17,348]. TGFβ signaling is not limited to the canonical Smad pathway, as they have also been reported to transduce their signal through non-Smad signaling pathways[14,32,42,44,54,354-356].

Breast cancer is the most commonly diagnosed cancer in women and triple-negative breast cancer is a sub-type of breast cancer lacking estrogen receptor, progesterone

receptor and Her2/neu. As such, triple-negative breast cancers are poorly responsive to current treatments and to date no clinically effective treatment is available.

MicroRNAs (miRNAs) are a novel class of small non-coding RNAs which have eluded researchers for decades stealthily regulating many of the major biological processes in eukaryotic cells by regulating their target genes post transcriptionally. These negative regulators of gene expression are capable of exerting pronounced influences on translation and stability of mRNA. TGFβ has been shown to regulate miRNAs. The role of miRNAs in the progression of breast cancer (BC) is emerging only recently. Several miRNA have been implicated in several steps of breast cancer progression (reviewed in[14]). We recently found TGFβ-mediated down regulation of miR-584 and up regulation of miR-181 to be critical for breast cancer cell motility[367,401]. Multiple miRNAs have been implicated in human diseases[368,369].

Of particular interest, the broadly conserved miRNA miR-30 family has been implicated in various human cancers. The miR-30 family is highly conserved in Vertebrates and is organized in three clusters on three different chromosomes. The miR-30 family has been implicated in many cellular processes from differentiation of adipocytes[402], B-cells[403] and osteoblasts[404], mammary gland lactation[405], cardiomyocyte autophagy[406] or in stemness[407]. In cancer, miR-30 has been implicated in medulloblastoma[408]. Up-regulation of miR-30 has been associated with metastasis in melanoma[409]. Recent studies have shown that miR-30 plays an important role in the mammary gland[405]. Decreased miR-30 levels have been reported in bladder cancer[410], whereas increased levels have been associated with resistant glioma[411]. miR-30 was shown to promote growth in glioma[412] and regulate EMT in pancreatic cells[413,414]. While the regulation of the miR-30 family members remain largely unknown, several reports indicated that miR-30 could be down-regulated by TGFβ in human colon cells[415], rat kidney cells[416].

Previous studies from our laboratory demonstrated that several miRNAs, including miR-181 and miR-584 could play an important role in mediating the TGFβ signaling pathways in human breast cancer [367,401]. Particularly, we investigated whether the miR-30 family of microRNAs could play a role in breast cancer with respect to TGFβ biological responses. We found that overexpressing miR-30 family affected the tumor suppressive arm of

TGFβ signalling with affecting the pro-metastatic arm. This finding linking miR-30 to the tumor suppressive functions of TGFβ and the widely observed down-regulation of miR-30 family in several cancers is of therapeutic interest.

3.3. Materials and methods

Cell culture and transfection

Human breast carcinoma SUM149PT were grown in F12 (Hyclone, Logan, UT, USA) supplemented with 5% FBS (Gibco, Grand Island, NY, USA), 2 mM L-glutamine (Hyclone) and penicillin/streptomycin (Hyclone), supplemented with hydrocortisone and insulin at 37 °C under a humidified atmosphere of 5% CO_2.

Transfections

Cells were transfected with different 100 nM miRNA mimics (Genepharma, Shanghai, China) or siRNA (Ambion, Life Technologies, Grand Island, NY, USA) using Lipofectamine^{TM} 2000 reagent (Invitrogen, Grand Island, NY, USA), according to the manufacturer's protocol.

Before treatment, SUM149PT cells were serum-starved for 24 h and stimulated with 100 pM TGFβ$_1$ (PeproTech) in DMEM supplemented with 2 mM L-glutamine. SUM149PT cells transfected with miRNA mimics or siRNA were transfected 48 h prior to TGFβ$_1$ treatment.

Cell Cycle Kinetics

SUM149PT cells were plated 10^5 cells per well in 24-well plates, starved overnight, and treated or not with 100 pM TGFβ in medium supplemented with 2% FBS but no hydrocortisone nor insulin for 24 h. Cells were washed with PBS then fixed in 70% ethanol for 2 h. Cells were then washed and resuspended in propidium iodide 50 $\mu g.m^{-1}L$, RNAse 50 $\mu g.mL^{-1}$, and Triton X-100 0.1%. A total of 10,000 nuclei were examined in an Accuri C6 flow cytometer (BD Biosciences, Mississauga, ON, Canada) and the software FlowJo.

Caspase 3/7 activity assay

SUM149PT cells were plated in 96-well clear-bottom solid white plates (Costar, Corning, NY, USA) and starved overnight. SUM149PT cells were treated or not with TGFβ (100 pM) in medium supplemented with 2% FBS for 48 h but no hydrocortisone and insulin. Caspase 3/7 activity was measured using the Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA) following the manufacturer's protocol. Briefly, cells were incubated in the presence of Caspase-Glo Reagent (Promega) for 2 h at bench temperature, and the luminescence was measured (EG± Berthold Luminometer).

Migration assay

SCP2 cells were grown in 6-well plates until confluency and serum-starved overnight. A scratch was generated in the cell monolayer in straight lines using a sterile 10 μL tip guided by a ruler. Cells were then stimulated with or without 100 pM $TGF\beta_1$ or 500 nM activin A for 24 or 48 h. Lines were drawn under the wells so photos could later be acquired from the same area. Photographs were taken initially and at the end of the ligand stimulation under phase contrast light microscopy (Olympus IX70, ImagePro AMS) and the wound closure were quantified by Image J software (NIH freeware).

Invasion assay

SCP2 cells were serum-starved overnight and seeded 5×10^4 onto a Matrigel-coated 24-well cell culture Transwell insert (8-μm pore size; BD Biosciences). Coating was done with 30 μl of 1:3 water-diluted growth factor reduced (GFR) Matrigel (BD) into each insert of the 24-tranwell invasion plate and incubated for 2 h at 37 °C in the cell culture incubator for Matrigel gelation. Cells were seeded in starvation medium on the top chamber the precoated Transwell Insert and were stimulated or not with 100 pM $TGF\beta_1$ for 24 h. The bottom chamber contained 10% FBS in DMEM medium which acted as chemoattractant. After 24 h, cells from the top chamber were removed by cotton swab and invading cells were fixed with 4% formaldehyde for 10 min and then stained with a crystal violet solution for 10 min. Images of the invading cells were photographed using an inverted microscope (Olympus IX70, ImagePro AMS) and total cell numbers were counted and quantified by Image J software (NIH freeware).

Real-Time-PCR

Total RNA was extracted using TRIzol reagents (Invitrogen). Reverse transcription of 250 ng total RNA using was carried out using on miScript reverse transcriptase (Qiagen, CA, USA) as the manufacturer's instructions in a Rotor Gene 6000 PCR detection system (Corbett, San Francisco, CA, USA). miRNA PCR thermoprofile conditions were as follows: 95 °C for 15 min, 40 cycles (94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s). mRNA PCR thermoprofile conditions were as follows: 95 °C for 3 min, 40 cycles (94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s) using iQ SYBR Green (BioRad, USA)

3.4. Results

3.4.1. Expression of miR-30 family members is inhibited by TGFβ in cancer cells of multiple origins

miRNA have been characterized as master regulators of the transcriptome. These small non-coding RNAs can regulate large proportions of the transcriptome by targeting the 3' untranslated region (3'UTR) of their cognate targets and exert broad biological responses. A single miRNA can affect the entire transcriptome of a cell[287]. Several miRNA have been implicated in human disease[368,369]. The broadly conserved miR-30 family has been associated with cell differentiation[402-404] and blastoma[408], melanoma[409], bladder cancer[410] and breast cancer[417]. The miR-30 is involved in breast tissue physiology[405] and angiogenesis[418]. Deregulation of miR-30 have been reported in bladder cancer and melanoma. The exact role of miR-30 in human cancer remains unclear. Indeed, miR-30 has been reported to act as metastamir in melanoma[409] and a tumor suppressor miRNA in breast carcinoma[417] and thyroid carcinoma[419]. Interestingly, miR-30 has also been implicated downstream of TGFβ signaling. Indeed, TGFβ was shown to down-regulate miR-30 in colon carcinoma[415]. The unclear role of miR-30 in TGFβ signaling prompted us to examine the regulation of the different miR-30 family members by TGFβ in cell lines of different origins shown in **Fig.3.1**. We used a set of breast cancer cell lines derived from pleural effusions (MCF7 early breast adenocarcinoma, MDA-MB-231 with greater metastatic potential, SCP2 with strong bone metastatic tropism derived from MDA-MB-231, SUM149PT triple-negative invasive ductal carcinoma and MCF-10A near diploid normal mammary epithelial cells). The effect of TGFβ on miR-30 levels was

assessed by real-time semi-quantitative PCR in cells stimulated or not with TGFβ for 24 h. As shown in **Fig.3.1A**, TGFβ down-regulated the expression of all miR-30 family members in the different breast cell lines tested. The ability of TGFβ to regulate miR-30 expression levels did not appear to be dependent on hormone receptor status of the cell lines tested. Interestingly, miR-30 levels were not affected in HuH7 cells, possibly due to a mutation in a downstream signal transducer in this cell line. We then investigated if the TGFβ inhibitory effect on miR-30 levels was restricted to breast tissue. As shown in **Fig.3.1B**, we found that TGFβ could also potently down-regulate miR-30 in a variety of human cancers including liver (HLE, HepG2), melanoma (WM115), colon carcinoma (Colo320DM), keratinocytes (HaCaT) and glioma (U87). This conserved regulation of miR-30 by TGFβ suggested an important role for miR-30.

We decided to investigate the role of miR-30 downstream of TGFβ by measuring the effect of miR-30 modulation on known TGFβ effects in breast cancer cells.

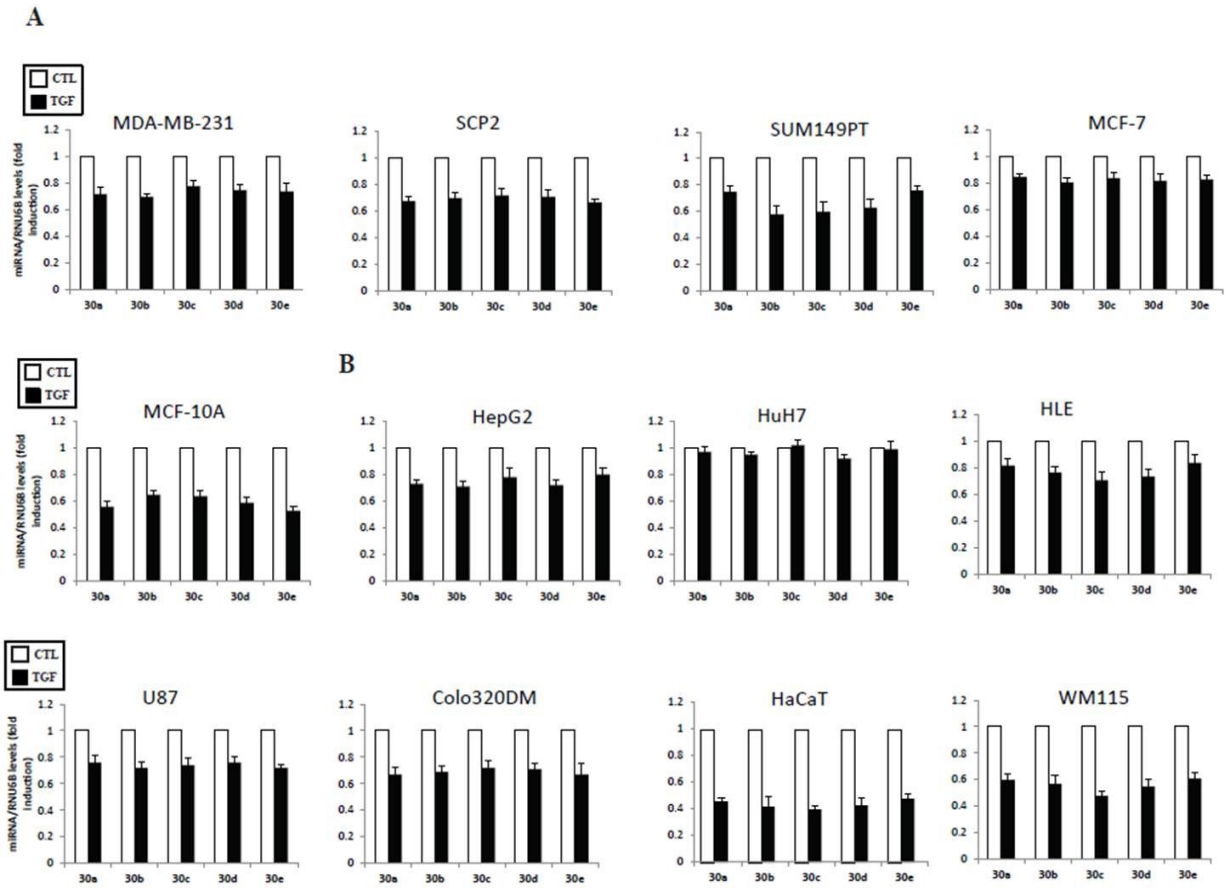


Fig.3.1: TGFβ down-regulated miR-30 in different tissues.

A, Triple negative breast cancer cell lines MDA-MB-231, SCP2, SUM149PT, early adenocarcinoma MCF-7 and normal immortalized MCF-10A cells were serum-starved overnight. Following a 24 h TGFβ stimulation, total RNA were isolated using Trizol and miR-30 levels were measured qRT-PCR and normalized to RNU6B spliceosomal RNA. **B**, Hepatocarcinoma (HepG2, HuH7, HLE), glioblastoma (U87), colorectal carcinoma (Colo320DM), squamous cell carcinoma (HaCaT) and melanoma (WM115) cells were serum-starved overnight. Following a 24 h 100 pM TGFβ1 stimulation, total RNA were isolated using Trizol and miR-30 were quantified by qRT-PCR and normalized to RNU6B. Error bars are SEM for 4 independent experiments.

3.4.2. miRNA-30 inhibits TGFβ-induced cell cycle arrest and apoptosis cytostatic effects

TGFβ acts as potent tumor suppressor in various target tissues, by inducing cell cycle, apoptosis and preventing cell immortalization[14,43,109,420]. The TGFβ tumor suppressive effects have been well described in breast cancer[42,381], hepatocarcinoma[76,353], pituitary tumors[351] and keratinocytes[43,382], among others. Moreover, previous reports indicated that miRNA modulation affected cell proliferation[380]. As we had observed that TGFβ broadly induced miR-30 inhibition, we decided to investigate the role of miR-30 downstream of TGFβ signaling. We first examined whether miR-30 could modulate the TGFβ tumor suppressive effects. We used different assays to assess miR-30 effects on the tumor suppressive response. We used highly TGFβ-responsive SUM149PT to assess the ability of miR-30 to modulate TGFβ-mediated cell cycle arrest using flow cytometry.

As shown in **Fig.3.2A**, TGFβ potently arrested triple-negative invasive ductal carcinoma SUM149PT cells in G1 is a dose-dependent manner. Indeed, an 80% increase of cells in G1 arrest was observed following increasing TGFβ stimulation ranging from 0.3 pM to 200 pM, indicating that TGFβ acts as a potent cell cycle inhibitor in these cells. As shown in **Fig.3.2B**, pretransfecting cells with a mix of double-stranded 2'O-methyl-modified miR-30 family sense sequences prior to TGFβ stimulation decreased the ability of TGFβ to arrest cells in G1. Indeed, the potent 80% increase of cells in G1 arrest following TGFβ observed in the control stimulation decreased to 20% in the miR-30 sense sequences pretreated condition, indicating that miR-30 acted as an inhibitor of TGFβ-mediated G1 arrest in SUM149PT.

TGFβ exerts strong pro-apoptotic effects in many tissues. Early in the study of miRNA, these small non-coding RNAs were shown to be major actors in apoptosis[421]. Recent

studies have identified pro-apoptotic[422] and anti-apoptotic[423] miRNA. Previous reports have indicated that miRNA modulation affected cell apoptosis[424]. Having observed an effect of miR-30 level modulation on one of the tumor suppressive effects of TGFβ, we tested the effect of miR-30 mimics on the pro-apoptotic effect of TGFβ. To characterize the role of miR-30, we used miRNA mimics again to increase miR-30 levels. As shown in **Fig.3.2C**, TGFβ potently induced apoptosis in SUM149PT cells in a dose-dependent manner. A gradual 75% increase of apoptotic cells was observed following a dose response of TGFβ stimulation ranging from 0.3 pM to 200 pM. As shown in **Fig.3.2D**, transfecting cells with miR-30 mimic sense sequences prior to TGFβ stimulation decreased the ability of TGFβ to induce apoptosis. Indeed, the 75% induction of apoptotic cells observed in the control condition decreased to 40% in the miR-30 mimic sense sequences pretreated condition, indicating that miR-30 acted as an inhibitor of TGFβ-mediated apoptosis in SUM149PT.

TGFβ exerts a strong inhibitory effect on immortalization in numerous cell lines[43]. In order to assess the effect of miR-30 on TGFβ-mediated inhibition of immortalization, we transfected SCP2 cells with an hTERT-lux reporter construct containing 2 kb of the hTERT gene promoter upstream of a luciferase gene[43]. As shown on **Fig.3.2C**, TGFβ induced an inhibition of luciferase activity indicating a decreased hTERT promoter activity reflecting the inhibitory effect of TGFβ on cellular immortalization. Transfecting the cells with miR-30 did not affect TGFβ-mediated hTERT promoter inhibition. Transient knock-down of TGFβ cytosolic effector Smad3 was used as a positive control[43]. This result suggests that miR-30 does not affect TGFβ-mediated inhibition of immortalization. It appears that miR-30 is necessary for some of the TGFβ tumor suppressive effects such as TGFβ-mediated G1 arrest and TGFβ-mediated apoptosis but not TGFβ-mediated inhibition of immortalization.

TGFβ has been extensively reported to have a tumor suppressive role in early stage breast cancer and to have a pro-metastatic role in late stage breast cancer. We observed a potent effect of miR-30 on some of the tumor suppressive effect of TGFβ signaling with an effect on TGFβ-mediated cell cycle arrest and apoptosis but not on the inhibition of immortalization. To further characterize the role of miR-30 on TGFβ biological effects,

we investigated the effect of miR-30 on known pro-metastatic effect of TGFβ. In later stage breast cancer, TGFβ has a strong cancer promoting effect in late stage breast cancer[401], which led us to investigate TGFβ-mediated migration and invasion in metastatic breast cancer.

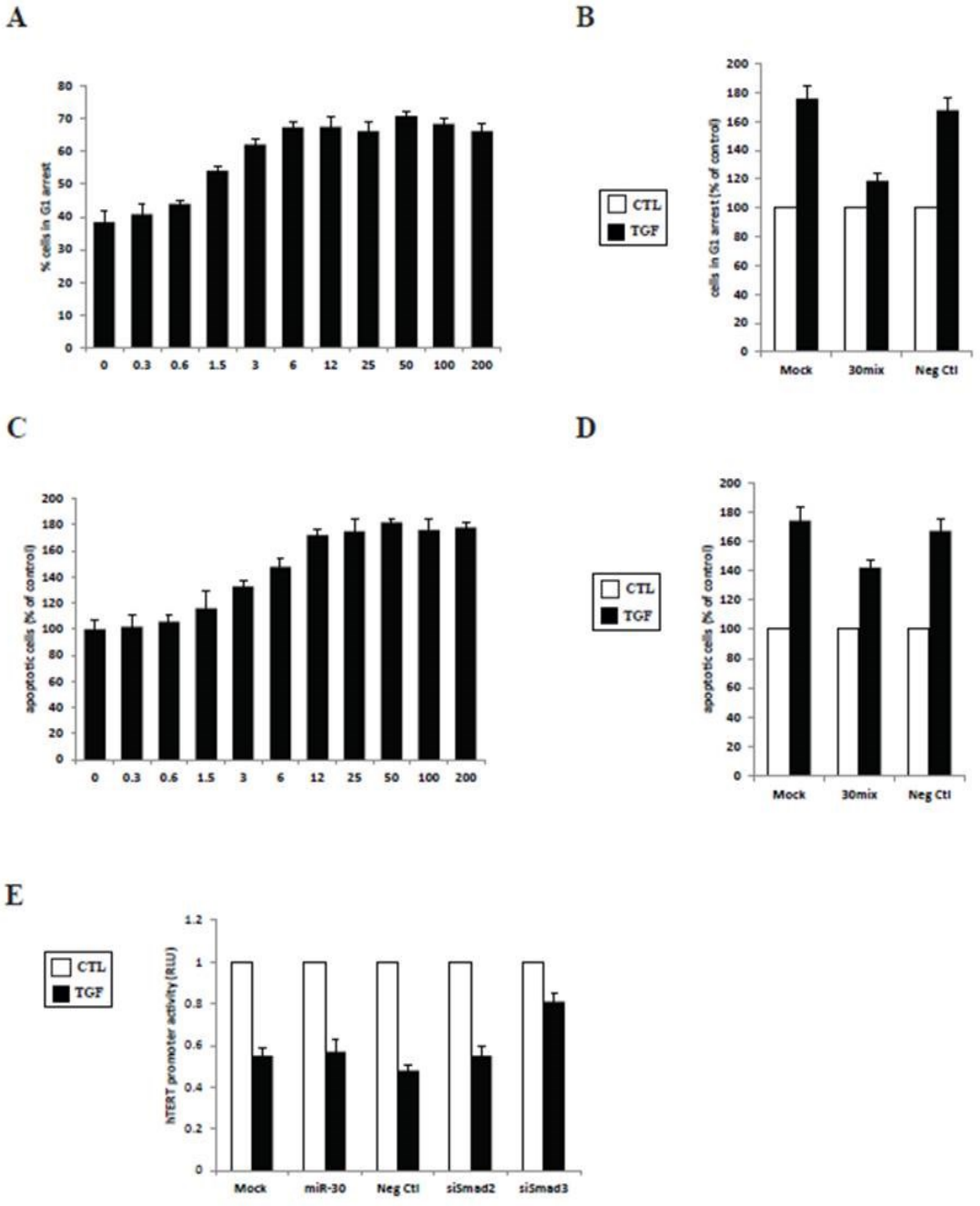


Fig.3.2: MiR-30 down-regulation is necessary for the anti-proliferative effect of TGFβ in SUM149PT.

A, TGFβ1 has a potent dose-dependent anti-proliferative effect on SUM149PT cells. SUM149PT cells were serum-starved overnight and stimulated with increasing concentrations of TGFβ1 for 24 h. An anti-proliferative effect could be seen at 1.5 pM and this effect reached a maximum at 6 pM TGFβ1. **B**, SUM149PT cells were transfected with 100nM miR-30 mimics (20nM mimic for each family member) and serum-starved overnight. Following 24 h TGFβ1 stimulation, a reduction of the TGFβ1 anti-proliferative effect as assessed by a decreased accumulation of cells G1 arrest effect was observed in miR-30 mimic transfected conditions but not in control conditions. **C**, TGFβ1 has a potent dose-dependent pro-apoptotic effect on SUM149PT cells. SUM149PT cells were serum-starved overnight and stimulated with increasing concentrations of TGFβ1 for 24 h. An pro-apoptotic effect could be seen at 3 pM and this effect reached a maximum at 12 pM TGFβ1. **D**, SUM149PT cells were transfected with 100nM miR-30 mimics (20nM mimic for each family member) and serum-starved overnight. Following 24 h TGFβ1 stimulation, a reduction of the TGFβ1 pro-apoptotic effect as assessed by a decreased caspase activity was observed in miR-30 mimic transfected conditions but not in control conditions.

3.4.3. TGFβ-mediated miR-30 down regulation is not required for TGFβ-induced cell migration/invasion in breast cancer cells

As miR-30 is strongly inhibited by TGFβ signaling in breast cancer, we further assessed the functional role of miR-30 in mediating TGFβ effects. TGFβ has a dual role in breast cancer, acting as a tumor suppressor in early carcinoma and later as a tumor promoter[14]. In late stage breast cancer and in other tissues, TGFβ no longer has a tumor suppressive role but exerts a pro-metastatic effect by modulation cellular migration and invasion. TGFβ has been shown to induce cellular migration and invasion in several tissues such as breast[401], prostate[425] and liver[162]. Having initially observed an effect of miR-30 level modulation on TGFβ tumor suppressive effects, we tested the effect of miR-30 on other known TGFβ pro-metastatic effects. To test the effect of miR-30 modulation on TGFβ pro-metastatic biological responses, we used SCP2, a highly metastatic breast cancer cell line in which TGFβ has a pro-migratory and pro-invasive effect. As shown in **Fig.3.3A**, TGFβ induced cell migration leading to further wound closure in SCP2 cells. Wound closure increased from 40% to 70% following 24 hours of TGFβ stimulation. Transfecting cells with miR-30 mimic sense sequences prior to TGFβ stimulation did not affect the pro-migratory effect of TGFβ. The pro-migratory effect of TGFβ appears independent of miR-30 levels.

We then used SCP2 cells to assess another important pro-metastatic effect of TGFβ. We assessed the effect of miR-30 over-expression on the ability of TGFβ to induce invasion through Matrigel. Transwell inserts were coated with basement membrane-mimicking

Matrigel and placed above medium containing 10 % FBS used as a chemoattractant. As shown in **Fig.3.3B**, TGFβ induced cellular invasion through Matrigel in SCP2 cells. The number of invading cells increased 8-fold following TGFβ stimulation. Transfecting cells with miR-30 mimic sequences prior to TGFβ stimulation, did not affect the pro-invasive response to TGFβ. The pro-invasive effect of TGFβ also appears independent of miR-30 levels.

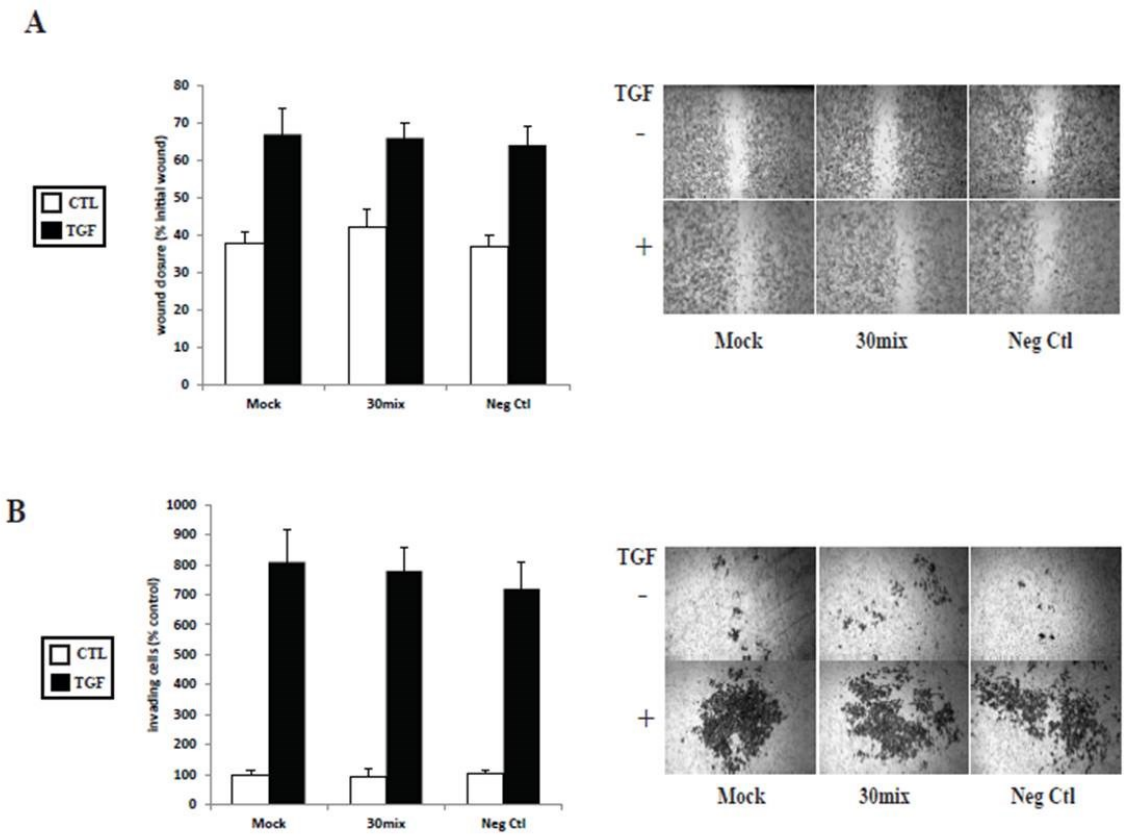


Fig.3.3: miR-30 down-regulation is not required for TGFβ1-induced migration and invasion in breast cancer.

A, A monolayer of highly metastatic triple negative SCP2 was wounded and cells were stimulated or not with 100 pM TGFβ1. The effect of ligand stimulation was visualized after 24 h using phase contrast microscopy. The area of the wound was measured using ImageJ software and the wound closure was graphed after normalization to the corresponding initial wound size. Data is graphed as the arithmetic mean of 4 independent experiments. The error bars are the standard error of the mean. **B**, SCP2 cells were pre-transfected with 100 nM miR-30 sense oligonucleotides. SCP2 cells were seeded in starvation medium on a Transwell cell culture insert coated with growth factor reduced Matrigel and stimulated or not with 100 pM TGFβ1 for 48 h. The invading cells were visualized and photographed on the bottom side of the filter of the insert after crystal violet staining using phase contrast microscopy. Representative photos are shown in the

right panel. Invading cells were counted using ImageJ software. Data is graphed as the arithmetic mean of 2 independent experiments. The error bars are the standard error of the mean.

Taken together, the above findings indicate that modulating miR-30 levels affected the tumor suppressive arm but not the tumor promoting arm of TGFβ signaling. In order to further our understanding of the miR-30 family, we investigated the contribution of individual members in the observed effects on the cell cycle and on apoptosis.

3.4.4. MiR-30 family members have varying effects on TGFβ-mediated G1 arrest and apoptosis

In order to further characterize the biological role of miR-30 downstream of TGFβ signaling, we tested the biological response of individual of the miR-30 family member overexpression. Although miRNA family members often have overlapping targets and are often jointly expressed, we tested whether specific members of miR-30 family inhibited TGFβ-mediated cell cycle arrest and apoptosis. We improved our understanding of the miR-30 by testing biological effects of individual miR-30 family members. The different miR-30 family members have a high sequence similarity and differ by as little as one base as miR-30a and miR-30d or miR-30a and miR-30e **Fig.3.4A**. In order to differentiate between different miR-30 family members, it was necessary to be able to modulate them individually. Shown in **Fig.3.4B**, transfecting cells with miR-30 2'O-methyl modified oligonucleotide mimics led to a strong increase in miR-30 family with a 6- to 20-fold increase in miRNA levels 72 hours post-transfection.

In order to functionally characterize the different miR-30 family members, we used specific miRNA mimics to increase individual miR-30 members. Overexpression of individual miR-30 family members mimics partially inhibited TGFβ anti-proliferative effects as assessed by G1 arrest in SUM149PT cells **Fig.3.4C**. The overexpression of the miR-30 family using a set of miRNA mimics more efficiently altered the TGFβ response. The improved inhibition of TGFβ effect by pooled miR-30 family mimics compared to individual members suggest a cooperative effect between different members of the miR-30 family. There is evidence for redundant miRNA target specificity within the same miRNA family[426,427] and smaller biological effects have been reported when the expression of only one miRNA family member was modulated[417].

To then investigate the role of the different miR-30 family members downstream of TGFβ-mediated apoptosis, we transfected cells with miR-30 family members mimics prior to TGFβ stimulation and measured caspase3/7 activity using Caspase-Glo luminescent reagent.

As shown in **Fig.3.4D**, transfecting breast cancer cells with individual miR-30 family members reduced the TGFβ-mediated apoptosis. In this case, increasing intracellular levels of the family member miR-30b, miR-30c and miR-30d resulted in a strong inhibition of TGFβ-mediated apoptosis from 70% to 30%. Transfecting cells with pooled miR-30 member sense sequences resulted in a lesser inhibition of TGFβ-mediated apoptosis from 70% to 40%. This suggests that there is no cooperative effect of different family members downstream of TGFβ-mediated apoptosis. The contribution of the effective miR-30b, miR-30c and miR-30d is decreased by the presence of the less effective miR-30a and miR-30e.

Taken together, it appears that miR-30 family members have different effects: miR-30a has a modest effect on both TGFβ-mediated cell cycle arrest and apoptosis, miR-30b affects mostly apoptosis, miR-30c and miR-30d have a stronger effect in both TGFβ-mediated cell cycle arrest and apoptosis and finally miR-30e mostly affects TGFβ-mediated cell cycle arrest. Taken together these observations suggest that all miR-30 family members are involved in TGFβ-mediated tumor suppression.

A

30a	UGU	AAA	CAU	CCU	CGA	CUG	GAA	G
30b					AC	C	AGC	U
30c					AC	C	UC	C
30d				C				
30e					U			

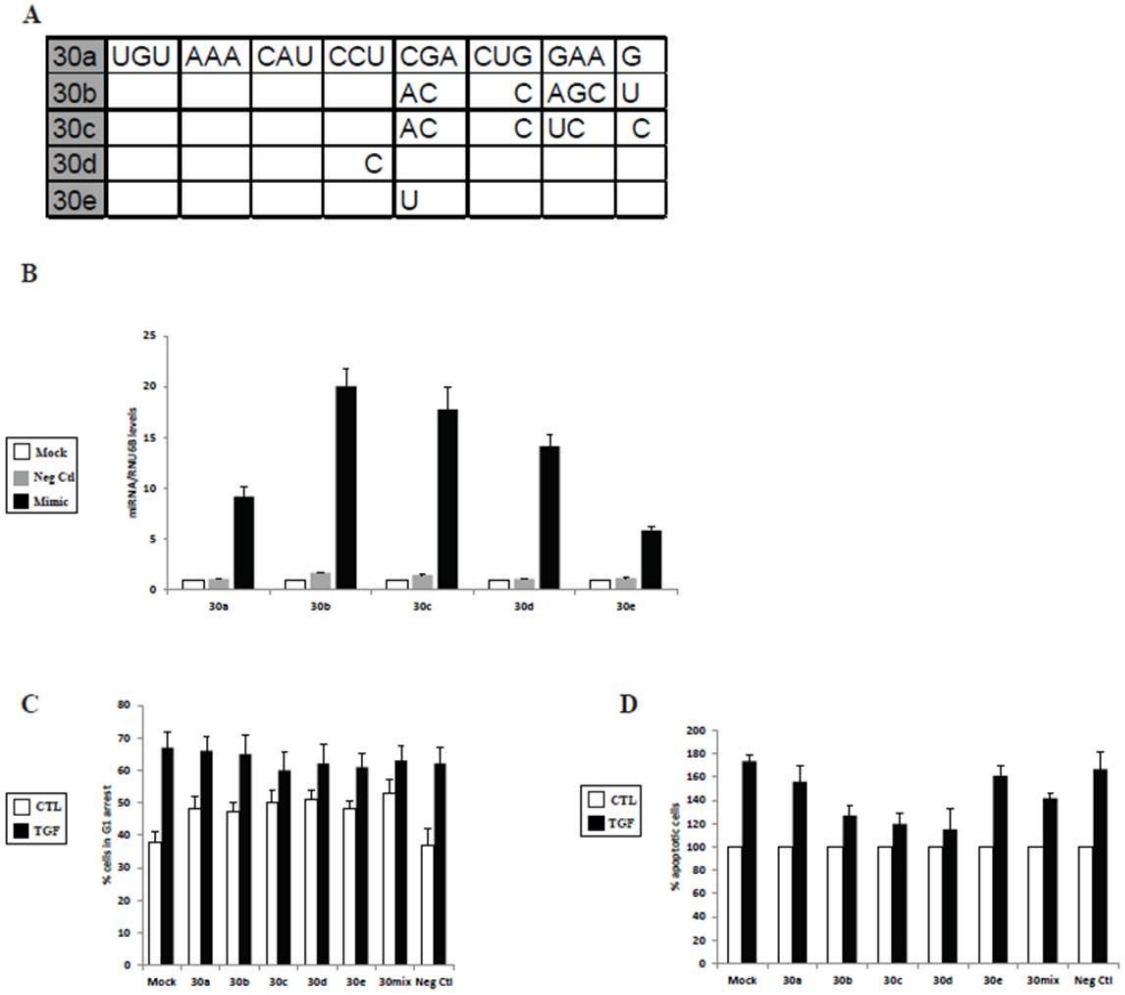


Fig.3.4: Effect of individual miR-30 members on TGFβ-mediated tumor suppression.

A, The miR-30 family members share a highly sequence similarity and differ by as little as one base pair. **B**, SUM149PT cells were transfected with 100 nM sense oligonucleotide and total RNA were isolated after 72 h using Trizol and miR-30 overexpression was measured by qRT-PCR and normalized to RNU6B. A potent overexpression of miR-30 family members was detected 72 h post-transfection. **C**, SUM149PT cells were transfected with 100 nM individual or pooled miR-30 family member mimic sense oligonucleotide and serum-starved overnight. Following a 24 h 100 pM TGFβ1 stimulation, a reduction of the TGFβ1 anti-proliferative effect as assessed by a decreased accumulation of cells G1 arrest effect was observed in miR-30 mimic transfected conditions. **D**, SUM149PT cells were transfected with 100 nM individual or pooled miR-30 mimics and serum-starved overnight. Following a 24 h 100 pM TGFβ1 stimulation, a reduction of the TGFβ1 pro-apoptotic effect as assessed by a decreased caspase activity was observed in miR-30 mimic transfected conditions but not in control conditions. Error bars are SEM of 3 independent experiments.

3.4.5. TGFβ-mediated tumor suppression requires different signaling pathways

To further analyze the molecular mechanisms by which TGFβ regulates miR-30, we examined the different signaling pathways known to mediate TGFβ signaling. The

canonical signaling pathway involves cytosolic receptor-associated Smad proteins which transduce membrane-associated receptor signaling into cytosolic and nuclear signaling[54]. The main transducers of TGFβ signaling are the cytoplasmic Smad proteins. As shown in **Fig.3.5A**, transient knock-down Smad2 but not Smad3 led to a decrease of TGFβ-mediated G1 arrest as assessed by a decrease of the number of cells in G1 of the cell cycle by flow cytometry. Indeed, the 25% increase in the number of cells in G1 arrest observed in the control conditions was decreased to 10% in the Smad2 knock down condition but no change was observed in the Smad3 knock down condition. This suggests that Smad2 is the cytosolic mediator of TGFβ signaling which leads cell to arrest G1 of the cell cycle. Smad3 does not seem to be mediating this cytostatic effect of TGFβ. This is interesting as Smad3 has a lower DNA-binding affinity compared to Smad2[428], which would suggest the presence of co-factors. Also Smad2 was shown to mediate TGFβ-mediated cytostatic effects in pancreatic cancer[429]. As some TGFβ-mediated signaling is transduced by non-canonical Smad-independent pathways, we decided to use chemical inhibitors of these pathways to selectively block signal transduction in order to assess their contribution to TGFβ-mediated G1 arrest. As shown in **Fig.3.5B**, using LY294002 (PI3 kinase inhibitor) resulted in a complete inhibition of TGFβ-mediated G1 arrest in SUM149PT cells. Similarly, pre-treatment with SP600125 (JNK inhibitor) led to a complete loss of TGFβ-mediated G1 arrest. Finally, to a lesser extent, pre-treatment with U0126 (MEK1/2 inhibitor) also resulted in a decreased TGFβ-mediated G1 arrest from about 2-fold in control conditions to 1.2-fold. SB431542 (TGFβ receptor type-I inhibitor or TβRI) was used as a positive control. Treatment with mTOR (Rapamycin) or p38 (SB203580) inhibitors did not affect TGFβ-mediated G1 arrest suggesting that these pathways are not involved in this biological response. These results suggest that the Akt/PI3 kinase, JNK and MEK/ERK pathways are also involved in the mediation of TGFβ-induced G1 arrest.

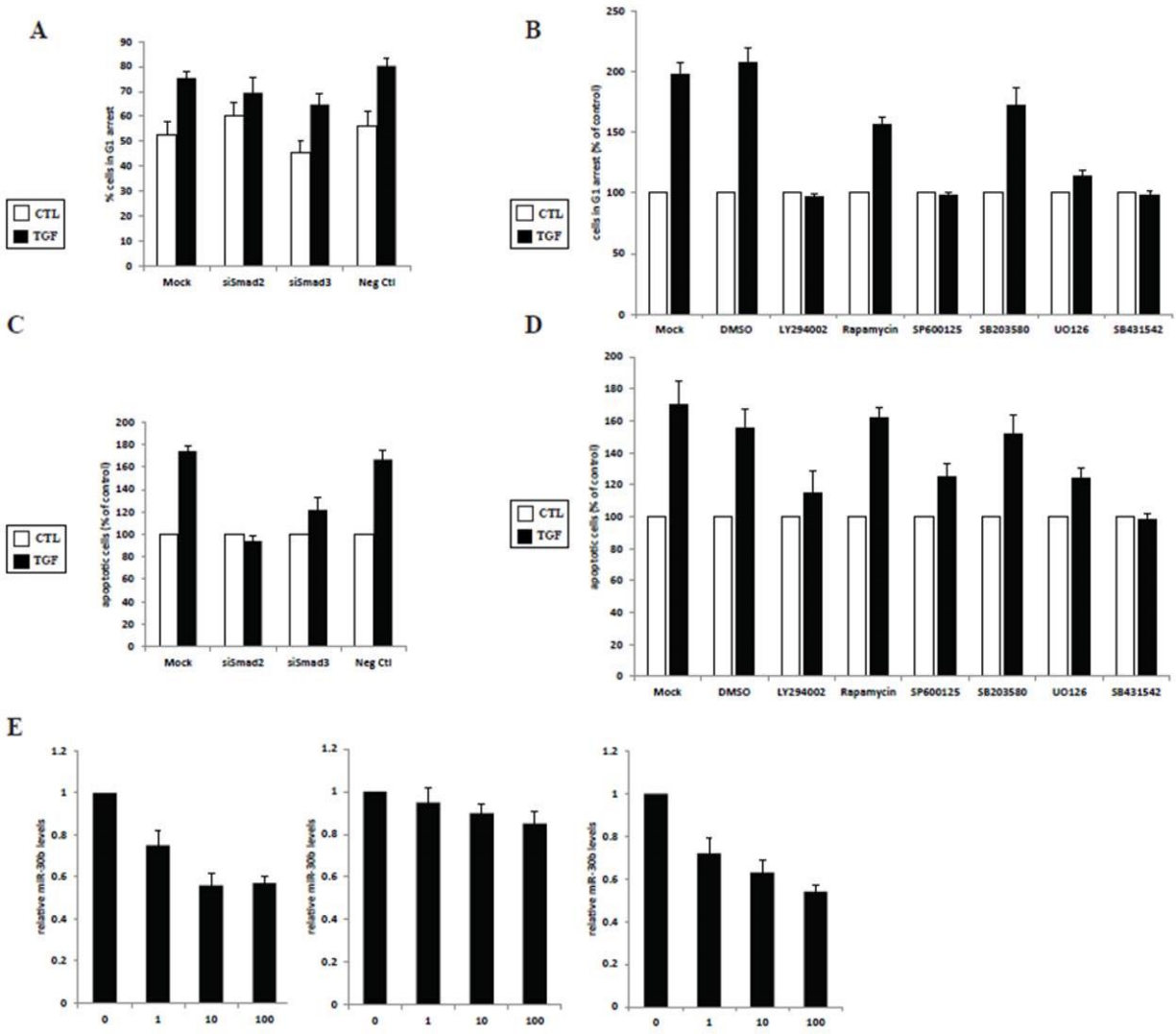


Fig.3.5: Role of canonical and non-canonical pathways in mediating TGFβ tumor suppression and miR-30 inhibition.

A, SUM149PT cells were transfected with 100 nM Smad2/3 siRNA or a negative control siRNA and serum-starved overnight. Following a 24 h 100 pM TGFβ1 stimulation, a reduction of the TGFβ1-induced anti-proliferative effect as assessed by a decreased accumulation of cells G1 arrest effect was observed in Smad2 knockdown condition but not in Smad3 knockdown or control condition. **B**, SUM149PT cells were serum-starved overnight and pre-treated with different chemical inhibitors then treated or not with 100 pM TGFβ1. TGFβ-mediated G1 arrest was assessed by FACS. DMSO was used as a vehicle control. **C**, SUM149PT cells were transfected with 80 nM Smad2/3 siRNA or a negative control siRNA and serum-starved overnight. Following a 24 h 100 pM TGFβ1 stimulation, a reduction of the TGFβ1-induced pro-apoptotic effect as assessed by a decreased caspase activity observed in both Smad2 and Smad3 knockdown conditions compared to control condition. **D**, SUM149PT cells were serum-starved overnight and pre-

treated with different chemical inhibitors then treated or not with 100 pM TGFβ1. TGFβ-mediated pro-apoptotic effect was assessed by luminescence of a caspase substrate. DMSO was used as a vehicle control. **E**, MiR-30 regulation is mediated through the TGFβ type-I receptor. SUM149PT cells were serum-starved overnight and pre-treated with type-I receptor inhibitor SB431542 (middle) or vehicle control (right) prior to being stimulated with a dose response of TGFβ1 (0, 1, 10 and 100 pM). The gradual TGFβ-mediated miR-30 inhibition (left) was abolished in SB431542-treated condition but not in control. Data is graphed as the mean of 3 independent experiments. The error bars are SEM of 3 independent experiments.

As above, we tested which receptor associated Smad protein transduced the TGFβ-mediated pro-apoptotic signal. We pretreated cells with siRNA individually targeting receptor-associated Smad2 and Smad3, serum-starved the cells and treated the cells with TGFβ. As shown in **Fig.3.5C**, knocking down Smad2 and to a lesser extent Smad3 led to decreased TGFβ-induced apoptosis. Indeed, the 75% increase in the number of cells undergoing TGFβ-induced apoptosis observed in the control conditions was abolished or decreased to 20% in the Smad2 and Smad3 knock down conditions respectively. This observation suggests that both Smad2 and Smad3 mediate the pro-apoptotic TGFβ signal. As mentioned above some TGFβ signal transduction is mediated by non-canonical Smad-independent pathways. To test the involvement of these pathways in TGFβ-induced apoptosis, we blocked these pathways using selective chemical inhibitors. As shown in **Fig.3.5D**, pre-treatment with LY294002 (PI3 kinase inhibitor), SP600125 (JNK inhibitor) and U0126 (MEK1/2 inhibitor) also resulted in a decreased TGFβ-mediated apoptosis in SUM149PT cells. Interestingly, the inhibition of TGFβ-mediated apoptosis was not completely abolished when PI3 kinase, JNK or MEK1/2 inhibitors were used. SB431542 (TβRI inhibitor) was used as a positive control. Treatment with mTOR (Rapamycin) or p38 (SB203580) inhibitors did not affect TGFβ-mediated apoptosis. Unlike in prostate cancer cells[430], TGFβ-mediated apoptosis appears independent of p38.

We observed that preventing TGFβ-induced miR-30 inhibition by transfection cells with miR-30 mimic sequences interfered both with TGFβ-induced G1 arrest and TGFβ-induced apoptosis. We also observed that both TGFβ-induced G1 arrest and TGFβ-induced apoptosis were mediated by cytosolic Smad effector proteins. We investigated the involvement of Smad proteins in the modulation of miR-30 levels downstream of TGFβ signaling. We pretreated cells with SB431542 after serum-starving the cells and

then stimulated the cells with TGFβ before collecting total RNA. TGFβ stimulation led to a 25% to 45% decrease of different miR-30 family members shown in Fig.1. MiR-30b was the most down-regulated member of the miR-30 family following TGFβ stimulation. We extracted total RNA and quantified miR-30b following TGFβ receptor inhibition. As shown in **Fig.3.4E**, TGFβ-mediated miR-30b inhibition was dose-dependent (left) and the gradual TGFβ-mediated inhibition of miR-30b was abolished using TβRI inhibitor (middle) but not when cells were treated with carrier (right). This indicates that TβRI is necessary for TGFβmediated miR-30 inhibition. Taken together, these results indicate that the canonical Smad pathway and several non-canonical pathways are involved in TGFβ-mediated tumor suppression and that miR-30 regulation is TGFβ receptor-dependent.

TGFβ is a potent regulator of cellular transcription, affecting the entire transcriptome. In order to characterize the biological effect of miR-30 overexpression, we investigated the modulation of the TGFβ transcriptional response following miR-30 overexpression in SUM149PT cells.

3.4.6. miR-30 modulates TGFβ tumor suppressive transcriptional response

We initially demonstrated the ability of TGFβ to act as a tumor suppressor in SUM149PT. TGFβ is a powerful regulator of several biological processes and a broad regulator of the transcriptome. Our data shows that TGFβ exerts a strong tumor suppressor effect in breast cancer cells and that miR-30 overexpression inhibits the TGFβ biological response suggesting that miR-30 modulates some of the TGFβ response genes. In order to identify TGFβ-regulated genes implicated in the tumor suppressive effect of TGFβ, we tested the effect of TGFβ stimulation on a subset of cell cycle- and apoptosis-related genes. These genes were selected from the Human Cell Cycle PCR array and Human Apoptosis PCR array (Qiagen). As shown in **Fig3.6A** and **Fig3.6B**, only about 20% of the genes tested we found to be regulated 1.5-fold following TGFβ stimulation. Among the genes involved in cell cycle, 6 out of 46 were induced by TGFβ more than 1.5-fold as shown in **Fig.3.6C**. Among the genes involved in apoptosis, 12 out of 52 were modulated by TGFβ: 6 genes were up-regulated and 6 were down-regulated following TGFβ stimulation as shown in **Fig.3.6D**. We identified 6 up-regulated genes with known

cell cycle involvement following TGFβ stimulation **Fig.3.6C**. Gadd45a is a major genotoxic stress sensor induced by DNA-damage. Gadd45a suppresses Ras-driven mammary tumors and induces apoptosis[431] and interacts with CDKN1A[432] and mediates G2/M checkpoint[433]. Gadd45a deficient cells are defective in UV-induced cell cycle arrest[434]. C-Myc-induced Chk1 was shown to induce cell cycle arrest and depletion of Chk1 affects caspase-dependent apoptosis[435]. We have recently shown CDKN1A (p21/Cip1) to be a TGFβ target in SUM149PT and to be involved in breast cancer cell migration and invasion[345]. MAD2L2 was shown to induce cell cycle arrest[436]. Depletion of MAD2L2 was shown to affect apoptotic responses[437]. E2F3 was also induced by TGFβ in our model cell line. We also identified 6 up-regulated and 6 down-regulated genes with known apoptosis involvement following TGFβ stimulation **Fig.3.6D**. Following TGFβ treatment, we observed a transcriptional induction of a subset of pro-apoptotic genes. APAF1, TNF, BCL2L11[438], CASP2[439], BCL2A1, DR6 (TNFRSF6) were induced. These two sets of genes can serve as quantitative indicators of the TGFβ tumor suppressive transcriptional response in SUM149PT cells.

As both miR-30 over-expression had been shown to interfere with TGFβ-induced G1 arrest and apoptosis, we investigated whether the effect of miR-30 on TGFβ signaling was mediated through an effect on TGFβ-regulated cell cycle- or apoptosis-related genes.

As miR-30 overexpression had decreased the ability of TGFβ to induce cell cycle arrest and apoptosis in SUM149PT cells, we tested the effect of miR-30 overexpression on these TGFβ-regulated cell cycle-related genes. To address the mechanism through which TGFβ-modulated miR-30 expression affects the tumor suppressive arm of TGFβ signaling, we searched for miR-30-modulated genes downstream of TGFβ signaling. In order to increase our understanding of the role of miR-30 downstream of TGFβ in breast cancer, we observed the effect of miR-30 on the TGFβ-regulated cell cycle and cell apoptotic genes as indicators of the TGFβ effect on cell viability. Having observed that miR-30 down-regulation was necessary for TGFβ signaling and TGFβ regulated a subset of target genes involved in cell cycle regulation and apoptosis regulation, we tested the effect of miR-30 on TGFβ target genes regulations. We pre-transfected SUM149PT cells with miR-30 sense sequences and then observed the effect of the resulting miR-30

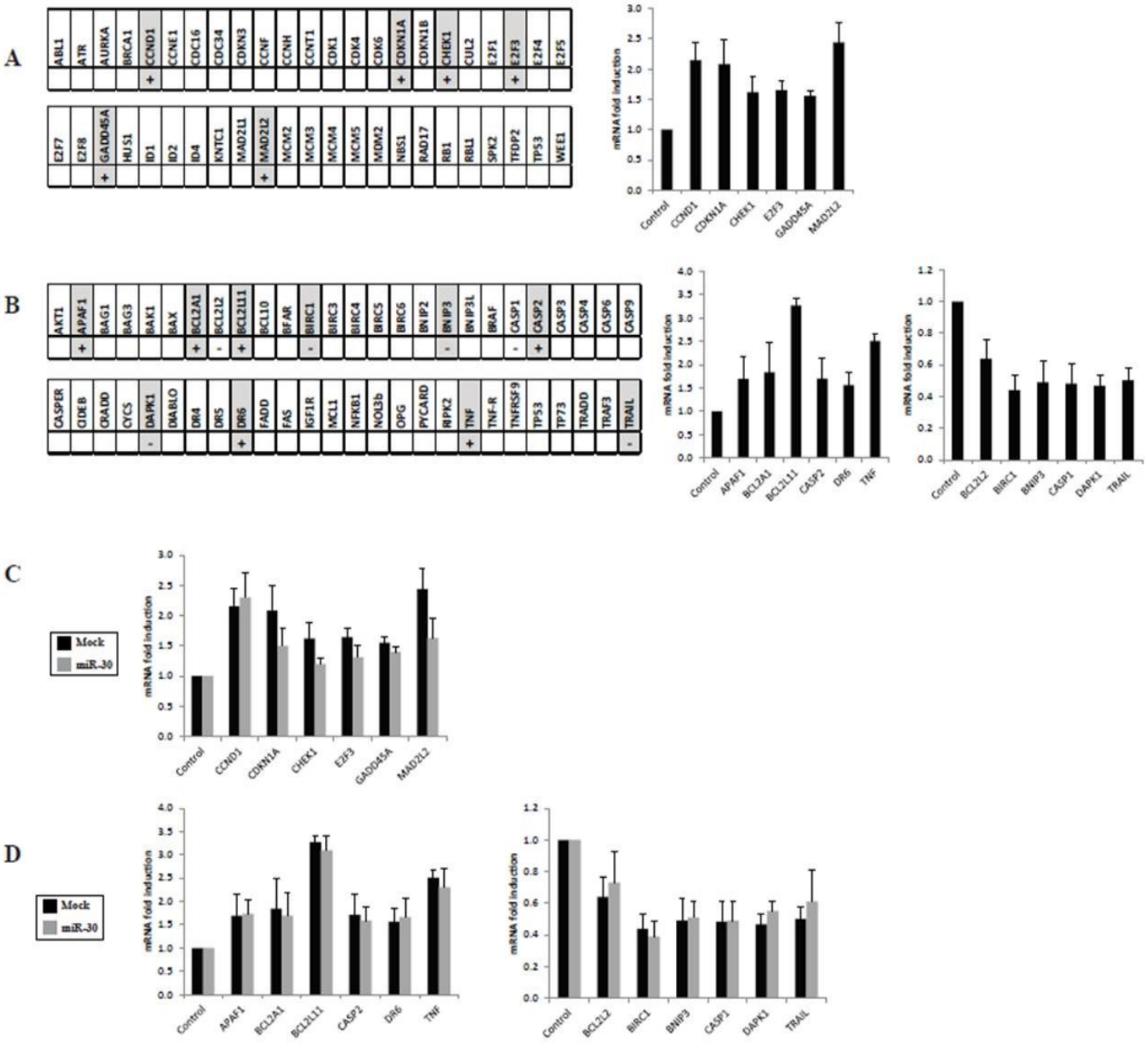
overexpression on TGFβ target gene regulation. As shown in **Fig.3.6E**, pre-treatment of SUM149PT cells with miR-30 mimics led to a decreased regulation of CDKN1A, CHEK1, E2F3 and MAD2L2 among the cell cycle-related TGFβ target genes and as shown on **Fig.3.6F** no apparent modulation of apoptosis-related TGFβ target genes was observed. This is contrast with the observed inhibitory effect of miR-30 on TGFβ-mediated apoptosis previously shown. This can be explained by a minor modulation of several apoptosis-related genes below our experimental sensitivity. Also other apoptosis-related genes can be affected by miR-30 effect. Interestingly, CDKN1A has recently been implicated downstream of miR-30a in colorectal carcinoma[440]. To confirm the role of these miR-30-modulated TGFβ target genes in cell cycle arrest in our model system, we used a transient knock-down approach. To assess the contribution of CDKN1A, CHEK1, E2F3 and MAD2L2 in mediating TGFβ effects on the cell cycle in SUM149PT, we disrupted these genes by transfecting cells with corresponding siRNA to knock-down endogenous mRNA levels prior to TGFβ stimulation. We then measured the proportion of cells in G1 of the cell cycle. As shown in **Fig.3.6G**, knocking down CDKN1A, CHEK1 and MAD2L2 but not E2F3 led to a decreased ability of TGFβ to arrest SUM149PT cells in G1 of the cell cycle. These findings suggest that CDKN1A, CHEK1 and MAD2L2 are targets of miR-30 and necessary for TGFβ-mediated cell cycle arrest.

This study highlights a novel role of miR-30 downstream of TGFβ signaling. The Smad-mediated TGFβ-induced miR-30 inhibition leads to a modulation of transcription levels of cell cycle-related miR-30 target genes. The TGFβ-mediated cell cycle arrest is modulated by miR-30 downstream of TGFβ signaling.

Fig.3.6: TGFβ-mediated cell cycle arrest is mediated in by miR-30 target genes CDKN1A, CHEK1 and MAD2L2.

The effect of TGFβ1 was tested on a pannel of cell cycle-related and apoptosis-related genes. SUM149PT cells were serum-starved overnight and stimulated for 24 h with 100 pM TGFβ1. Total RNA were extracted using Trizol and TGFβ-regulation of cell cycle-related (**A**) and apoptosis-related (**B**) genes was assessed by qRT-PCR. Six cell cycle-related genes were up-regulated by TGFβ1, and among apoptosis-related genes six were up-regulated and six down-regulated. Pre-transfecting SUM149PT cells with miR-30 sense oligonucleotides altered the TGFβ transcriptional response. MiR-30 inhibited the TGFβ-mediated induction of some cell cycle-related (**C**) genes such as CDKN1A, CHEK1 and MAD2L2, but not apoptosis-related (**D**) genes. **E**, CDKN1A, CHEK1 and MAD2L2 are necessary in TGFβ-mediated cell cycle arrest. SUM149PT cells were pre-transfected with 100 nM CDKN1A, CHEK1, MAD2L2 or control siRNA and serum-starved overnight prior to 24 h 100 pM TGFβ1 stimulation. Cell cycle distribution indicated that

CDKN1A, CHEK1, MAD2L2 knockdown resulted in decreased TGFβ-mediated cell cycle arrest compared to negative control. Error bars are SEM of 3 independent experiments.



3.5. Discussion

Because of their ability to simultaneously regulate multiple target transcripts, miRNA are able to affect several biological processes[261,262]. Consistent with our findings, miR-30 has been shown be induced during senescence and to act as a tumor suppressor[441]. Other studies linked miR-30 with epithelial-mesenchymal transition[413,419]. MiR-30 has also been associated with a metastatic signature in several cancers including breast[442]. Interestingly, it was recently shown that miR-30 family members were down-regulated in ER- and PR- tumors, suggesting that miR-30 expression was under hormonal control[303]. This was confirmed by the recent observation that miR-30 family was down-regulated by progestins[443]. Other recent studies on miR-30 have suggested miR-30 targets the ubiquitin pathway and the actin cytoskeleton pathway[444]. MicroRNAs are of great therapeutic interest[14]. We showed that miR-30 was a target of TGFβ in numerous breast cancer cell lines and in other tissues. This finding is consistent with previous findings of miR-30 down-regulation by BMP2 in murine bone marrow cells[404] and human smooth muscle cells[445]. We observed an effect of miR-30 on the TGFβ tumor suppressive response as assessed by both cell cycle arrest and apoptosis. We did not find any effect of miR-30 modulation on the TGFβ inhibitory effect on immortalization. We identified a subset of pertinent cell cycle-related and apoptosis-related TGFβ-target genes which were modulated by miR-30 overexpression. These pertinent genes could serve as downstream indicators of TGFβ tumor suppressive activity. In this study, we describe a tumor suppressor role for the miR-30 family downstream of TGFβ signaling in human breast cancer progression. TGFβ had been shown to down-regulate miR-30 in human colon[415] and murine mammary tumors[343]. Two recent studies showed that miRNA-mediated changes in protein expression were usually preceded by changes in mRNA expression, indicating that mRNA degradation is a key component of mammalian miRNA function[261,262]. These findings give strong support to our target identification strategy in SUM149PT cells.

We initially demonstrated the ability of TGFβ to potently arrest SUM149PT in the G1 phase of the cell cycle in a dose-dependent manner. We demonstrated that this effect was mediated through Smad2 and not Smad3. This finding contrasts with previous work

suggesting that Smad2 mediated the TGFβ migratory response while Smad3 mediated the TGFβ tumor suppressive response[429]. As Smad2 has lower DNA-binding affinity relative to Smad3, this suggests the presence of coactivators. We then investigated whether the TGFβ non-Smad signaling pathways were also involved in the TGFβ-mediated growth arrest, by specifically inhibiting these pathways using chemical inhibitors in the presence of TGFβ (LY294002 for PI3Kinase, Rapamycin for the mTOR in the PI3K/Akt pathway, U0126 for MEK1/2 in the MAP Kinase pathway, SB203580 for the p38 and SP600125 for the JNK in the p38/JNK pathway) As a positive control we also used the TGFβ type I receptor kinase inhibitor SB431542. SB431542 was characterized as a potent inhibitor of the activin, TGFβ and nodal type I receptors (ALK4, ALK5 and ALK7 respectively)[378]. Our findings suggest that different non-Smad pathways are involved in the TGFβ-mediated growth arrest, possibly through a negative feedback mechanism affecting the canonical Smad pathway. Indeed, inhibitors of the final mediators of the different pathways did not alter TGFβ-mediated cell cycle arrest but inhibitors of upstream effectors involved in negative feedback mechanisms altered the TGFβ response.

We have shown that TGFβ signaling leads to a decrease of endogenous miR-30 levels and that miR-30 down-regulation is mediated through TβR and is necessary for Smad-mediated tumor suppressive effects on cell cycle and apoptosis. We have shown that TGFβ induces miR-30-modulated target genes CDKN1A, CHEK1, MAD2L2 involved in the cell cycle. MAD2L2 encodes the key spindle checkpoint protein MAD2L2, part surveillance system which delays anaphase until all chromosomes are correctly oriented. Defects in this mitotic checkpoint are known to contribute to genetic instability which is associated to solid cancer progression[446]. This is the first report of MAD2L2 as a TGFβ-induced target gene. MAD2L2 levels have been associated with poor prognosis in colorectal cancer[446] and neuroblastoma[447], where it was shown to be an E2F1 target. CHEK1 is part cytosolic kinase that prevents entry into mitosis. CHEK1 is expressed at lower levels in triple negative breast cancer cells[448]. CHEK1 has been shown to be critical for proliferation in triple negative breast cancer cells[449], and its expression levels to be independent of p53 and Rb function[449]. We identified CHEK1 as a novel TGFβ target gene. To our knowledge, this is the first report of CHEK1 being induced by TGFβ. The

induction of these target genes can be a direct transcriptional effect and also indirect through a down-regulating miR-30 which removes a transcriptional inhibition. Importantly, we identified MAD2L2 and CHEK1, two novel TGFβ target genes, which are necessary for TGFβ-mediated cell cycle arrest in triple negative breast cancer cells.

This study brings further understanding on the role of miR-30 target genes involved in the cell cycle downstream of TGFβ signaling and brings important insight into the potential effects of miR-30 modulating therapies. Improving our understanding of the effect of miRNA on the transcriptome will bring new therapeutic avenues cancer treatment.

3.6. Acknowledgments

The authors are thankful to Dr. Stephen P Ethier, Dr. Joan Massagué, Dr Louise Larose for kindly providing us SUM149, SCP2 and WM115 cell lines respectively. This work was supported by grants from the Canadian Institutes for Health Research (CIHR) to JJL. JJL is the recipient of the McGill Sir William Dawson Research Chair.

Grant Support

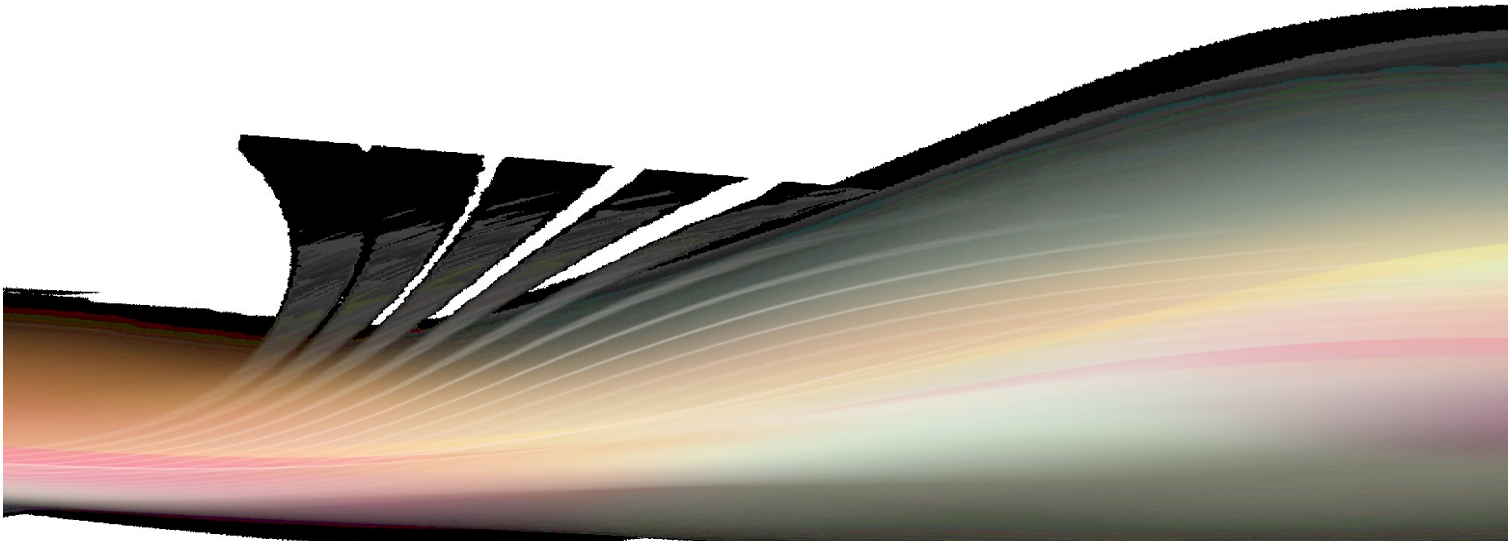
This work was supported by a Canadian Institutes for Health Research (CIHR) grant (fund code 230670 to JJL). JJ Lebrun is the recipient of a Sir William Dawson McGill Research Chair.

Having successfully identified several TGFβ-regulated miRNA mediating both TGFβ pro-metastatic[367,401] and tumor suppressive effects, we broadened the scope of our work to the TGFβ-regulated fraction of the miRNome. We have identified miRNAs which were up-regulated or down-regulated by TGFβ and which were necessary for TGFβ-mediated cellular responses. With the intention of identifying several druggable TGFβ target miRNAs in triple negative metastatic breast cancer, we performed a broad screen in a highly metastatic model cell line. We characterized a subset of miRNAs of interest downstream of TGFβ.

Chapter 4

TGFβ-regulated microRNA and their role downstream of TGFβ signalling in breast cancer

Jean-Charles Neel, Jean-Jacques Lebrun*



4.1. Abstract

TGFβ is a pluripotent cytokine that play important roles in embryonic development, cell proliferation, apoptosis, immortalization and cancer progression[14,17,43,108,109,344]. TGFβ potently reshapes the transcriptome by modulating numerous transcription factors, which then change the expression levels of hundreds of genes. MicroRNAs (miRNAs) are a novel class of small non-coding RNAs, which have eluded researchers for decades stealthily regulating many of the major biological processes in eukaryotic cells by regulating hundreds of their target genes post transcriptionally. MiRNAs have been implicated in many cellular processes such anchorage-independent growth, response to radiotherapy and angiogenesis. These non-coding RNAs are considered master regulators of the transcriptome and a subset of these RNAs can regulate a large proportion of the transcriptome. In order to understand the role of miRNA downstream of TGFβ signaling, we identified a subset of TGFβ-regulated miRNAs in breast cancer and attempted to further characterize the response associated with these regulations. We modulated endogenous miRNA levels by transfecting exogenous synthetic sense or antisense sequences and observed changes in the cellular response to TGFβ.

4.2. Introduction

TGFβ is multifaceted cytokine that has been shown to play a central role in a range of biological processes from embryonic development, cell proliferation, apoptosis, immortalization to cancer progression [14,17,43,108,109,344]. The founding member of this family is TGFβ and its cognate receptors are expressed throughout the body and their deregulation have been associated in numerous diseases [20]. TGFβ has been shown to regulate both tumor suppression and tumor progression in breast cancer. The ligand initially inhibits epithelial cells proliferation and early-stage cancer cells, but later facilitates cancer progression to metastasis [54,345-347,420]. TGFβ can be produced and secreted into the microenvironment by cancer cells and stromal cells alike. High intratumor levels of TGFβ correlate with higher histological grade, risk of metastasis, poor response to chemotherapy, and overall poor patient prognosis[346]. TGFβ binds to and signals through two serine/threonine kinase receptors (TβRI/ALK5 and TβRII), which

subsequently phosphorylate cytosolic Smad proteins (Smad2 and 3) [14,17,348]. TGFβ signaling is also mediated through Smad-independent pathways [32,42,44,54,354-356].

MicroRNAs (miRNAs) constitute a new category of small non-translated RNAs which have so far eluded researchers while stealthily modulating numerous major biological processes in eukaryotic cells by affecting their target genes post transcriptionally. These miRNA act as master regulators of the transcriptome and drastically influence translation and stability of target mRNA. Our limited understanding of miRNA has grown remarkably since its first observation in worms [263] to a fully accepted new class of regulatory molecules. Though the effect of TGFβ on mammary gland development and breast cancer extensively studied, the role of miRNA in this tissue is just starting to be explored. Bioinformatics approaches indicate that miRNAs comprise 1% of all human genes and regulate one third of the transcriptome [260]. Clearly miRNA play a central and critical role in disease and including cancer. Half of the miRNAs are on fragile chromosomal sites and these breakpoints suggesting that they could play a key role in tumorigenesis [299]. Often breakpoints coincide with fragile chromosomal sites [300]. Many miRNA-encoding genes are in chromosomal regions altered during tumorigenesis [301]. TGFβ regulates miRNAs. Their role in breast cancer (BC) progression is starting to emerge. Some miRNA are involved in different steps of breast cancer progression (reviewed in Humbert *et al.* 2010[14]). We recently found TGFβ-mediated down regulation of miR-584, miR-30 and up regulation of miR-181 to be critical for breast cancer cell motility [367,401]. Multiple miRNAs have been implicated in human diseases[368,369].

Several miRNA studies downstream of ligand signaling were conducted in breast and liver cancer leading to the characterization of ligand-regulated miRNAs. This prompted us to investigate TGFβ-regulated miRNAs downstream of TGFβ signaling. Such miRNA could possibly further the understanding of TGFβ signaling in the context of highly metastatic breast cancer. Triple-negative breast cancers are a TGFβ-sensitive subtype of breast cancer which can become highly metastatic and for which no therapeutic strategy has been effective. Improving our understanding of the miRNA-mediated biological response to TGFβ could lead to new therapeutic targets or therapeutic strategies

4.3. Material and methods

Cell culture

Human breast carcinoma MDA-MB231, SCP2, SCP3, MCF7 were grown in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 2 mM L-glutamine (Hyclone) and penicillin/streptomycin (Hyclone) at 37 °C under a humidified atmosphere of 5% CO_2. HuH7 and HaCaT cells were grown in the same conditions. WM278 cells were grown in RPMI (Hyclone) in similar conditions.

Microarrays

Cells were grown to confluence serum-starved and stimulated or not with 100pM TGFβ1 for 24 h. Total RNA were collected and purified using miRNeasy columns from Qiagen (CA). RNA samples were send to LC Bioscience (CA) where Affimetrix microarray were performed and alanlyzed. Samples were analyzed in simplicate.

Transfections

Cells were transfected with different 100 nM miRNA mimics and inhibitors (Genepharma, Shanghai, China) or siRNA (Ambion, Life Technologies, Grand Island, NY, USA) using Lipofectamine^{TM} 2000 reagent (Invitrogen, Grand Island, NY, USA), according to the manufacturer's protocol.

Before treatment, MDA and SCPs cells were serum-starved for 24 h and stimulated with 100 pM TGFβ_1 (PeproTech) in DMEM supplemented with 2 mM L-glutamine. SCP2 cells transfected with miRNA mimics or inhibitors were transfected 48 h prior to TGFβ_1 treatment.

Real-Time-PCR

Total RNA was extracted using TRIzol reagents (Invitrogen). Reverse transcription of 250 ng total RNA using was carried out using on miScript reverse transcriptase (Qiagen, CA, USA) as the manufacturer's instructions in a Rotor Gene 6000 PCR detection system (Corbett, San Francisco, CA, USA). miRNA PCR conditions were as follows: 95 °C for 15 min, 40 cycles (94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s).

Cell viability assay

Following an overnight FBS deprivation, HuH7 or HaCaT cells were resuspended (1×10^5 cells.ml^{-1}) in DMEM supplemented with 2% FBS in the presence or absence of 100 pM TGFβ_1 and seeded (1×10^4 cells.ml^{-1}) in 96-well plate for 48 h. After 48 h, cells were incubated for 2 h with Thiazolyl blue tetrazolium bromide **(**MTT) (5 mg.ml^{-1} in PBS, Sigma-Aldrish, Oakville, ON, Canada). Then a 20% SDS solution in 50% Dimethyl-formamide in H_2O at pH 4.7 was added to stop the reaction and mixed to homogeneity by pipetting. The absorbance at 570 nm was measured on a plate reader (BioTeK PowerWave XS) and a 690 nm measurement was used as a reference.

Migration assay

Cells were grown in 6-well plates until confluency and serum-starved overnight. A scratch was generated in the cell monolayer in straight lines using a sterile 10 µL tip guided by a ruler. Cells were then stimulated with or without 100 pM TGFβ_1 or 500 nM activin A for 24 or 48 h. Lines were drawn under the wells so photos could later be acquired from the same area. Photographs were taken initially and at the end of the ligand stimulation under phase contrast light microscopy (Olympus IX70, ImagePro AMS) and the wound closure were quantified by Image J software (NIH freeware).

Cell Cycle Kinetics

SUM149PT cells were plated 10^5 cells per well in 24-well plates, starved overnight, and treated or not with 100 pM TGFβ in medium supplemented with 2% FBS but no hydrocortisone nor insulin for 24 h. Cells were washed with PBS then fixed in 70% ethanol for 2 h. Cells were then washed and resuspended in propidium iodide 50 µg.m^{-1}L, RNAse 50 µg.mL^{-1}, and Triton X-100 0.1%. A total of 10,000 nuclei were examined in an Accuri C6 flow cytometer (BD Biosciences, Mississauga, ON, Canada) and the software FlowJo.

4.4. Results

4.4.1. TGFβ regulates a large subset of microRNA in breast cancer

TGFβ is known to have a dual role in breast cancer with an initially prominent tumor suppressor role that is later lost while tumor promoting effects are retained[54]. TGFβ modulates largely modulated the transcriptome and regulated a range of biological processes. The small non-coding miRNA have been shown to broadly affect the transcriptome (heart-specific miRNA in brain cells, or both SILAC papers). As TGFβ has been shown to modulate a spectrum of biological responses and since miRNA have also been implicated in an array of biological processes, we investigated the role of TGFβ on the miRNome. For this, we stimulated a panel of breast cancer cells with TGFβ for 24 hours and collected total RNA and assessed the miRNome by miRNA microarray and compared it to non-stimulated controls. As shown in **Fig.4.1A**. Several miRNA were modulated by more than 1.5-fold following TGFβ stimulation. Interestingly, TGFβ-mediated miRNA regulation appeared conserved across breast cell lines. Other studies[339,343,450] had assessed the effect of TGFβ stimulation on miRNA levels in breast, liver and gastric cells and had produced a short-list of TGFβ-regulated miRNA that largely matched our own as shown in **Fig.4.1A**.

In order to confirm the TGFβ-mediated miRNA regulation microarray data, we assessed miRNA levels by qRT-PCR following TGFβ stimulation. We stimulated cells with TGFβ and extracted total RNA using Trizol and performed qRT-PCR on 3 ng RNA and used RNU6B as an internal control. Our qRT-PCR data shown in **Fig.4.1B** largely matched our microarray data however the extent of TGFβ-mediated miRNA regulation varied between qRT-PCR and microarray measurements. Interestingly, TGFβ-mediated miRNA modulation did not appear to correlate with tumor aggressiveness as numerous miRNA were found to be regulated in both highly metastatic cell lines such as MDA-MD-231, SCP2 and SCP3 and less metastatic cell lines such as MCF-7 or even normal MCF-10A. TGFβ-induced miRNA modulation appears to be conserved in breast tissue. No statistical analysis was performed and only a subset of miRNA were further analyzed..

Based on microarray and qRT-PCR data, we characterized as being UP or DOWN regulated. In order to further characterize the role of these TGFβ-regulated miRNA

downstream of TGFβ signaling in breast cancer, it was necessary to modulate endogenous levels.

4.4.2. TGFβ-regulated microRNA and their role TGFβ-mediated tumor suppression

As TGFβ acts as a potent tumor suppressor in early stage breast carcinoma, we decided to assess the potential role of TGFβ-regulated miRNA downstream of TGFβ in the mediation of TGFβ tumor suppressive effects. For this we initially assessed the cytostatic effect of TGFβ after modulating endogenous TGFβ target miRNA. For up-regulated miRNAs, we pre-transfected cells with 100 nM miRNA inhibitors or antisense oligonucleotides. For down-regulated miRNA, we pre-transfected cells with 100 nM miRNA mimics or sense sequences. This interference on miRNA levels was intended to counter the TGFβ modulation observed previously. As HuH7 had similar TGFβ target miRNA, and is highly responsive to the TGFβ cytostatic effects, we used this cell line. Hepatocarcinoma cells were transfected with miRNA mimics or inhibitors to modulate endogenous miRNA levels prior to serum-starvation. After TGFβ stimulation, cell proliferation was assessed by MTT assay and the cytostatic effect of TGFβ was determined by comparing the absorbance in TGFβ-treated versus control conditions. We used Smad2 and Smad3 siRNA as a positive control as Smad3 was shown to mediate part of TGFβ cytostatic effects. As shown in **Fig.4.2A**, when endogenous miRNA levels were modulated prior to TGFβ stimulation, we did not observe any significant change in cell proliferation in HuH7 cells after 72 hours. We performed the same experiment in MCF-7 and HaCaT cell lines and did not observe any effect of miRNA modulation on TGFβ-mediated cytostatic effects (data not shown). This lack of effect of any of the TGFβ-regulated miRNA suggested than the cytostatic effect of TGFβ was mediated by non-miRNA pathways.

A, Breast cancer cell lines MCF-7, SCP2, SCP3, MDA-MB-231 were treated or not with 100 nM TGFβ1 for 24 hours and miRNA levels were assessed by Affimetrix miRNA microarray and TGFβ fold inductions were normalized to non-stimulated control. Data from other studies were added for reference : HuH7[339], NMuMG[343], BCG823[450]. **B**, TGFβ-mediated miRNA regulations were confirmed in SCP2 cells stimulated or not with 100 nM TGFβ1 for 24 hours. MicroRNA levels were quantified by qRT-PCR and TGFβ-induced fold regulations were determined by normalizing miRNA levels in TGFβ to non-stimulated controls. To the right, TGFβ-induced miRNA are labelled "up" and TGFβ-inhibited miRNA are labelled "down".

A

	Microarray Data						
	MCF7	SCP2	SCP3	MDA-231	HuH7	NMuMG	BGC823
181a		1.25	1.13	1.45	2.07		
181b		1.75	1.48	1.29			0.58
181c		1.43		1.70	2.29		0.59
181d	0.69	1.64	1.43		2.27		
181a2*		6.35		3.11			
200a	1.28				2.14		
200b	0.89	2.70	1.73	1.83	1.72		
200c						0.32	
23a		1.22	1.14	1.29	2.63		0.56
24					2.05		
27a		0.75	0.88		1.89		1.71
30a		1.19	0.76	1.25			0.57
30b	0.83			0.81			
30c	0.82			0.88		0.81	0.59
30d			0.71	1.18		0.71	
30e	0.82	1.48	0.58	1.19		0.67	
19a			0.26	1.46			
19b			0.47	1.36			
31						0.81	
21					1.58	1.64	
182		0.73	1.56	0.80			
18a		0.54	0.66				
18b		0.56	0.66	1.34			
143				9.07			
374b		1.84	1.52	0.77			
374a	0.51			0.40			
877			0.68				
92a	1.10	1.15	1.12				
92b	1.17	1.42	1.31				0.63
145				7.85			
584		0.50		0.75			
151-3p				1.27			
151-5p		1.32	1.28	1.19			
138-1*							
34a	0.78	0.38					0.60
365	0.86	6.19		1.57			
17		0.85		1.16			
20a		0.78	0.84	1.19	0.49		
186*	0.11		0.04	0.14			
877	0.59			0.39			
513-5p	0.67	8.10					
130a		0.47	0.60				
130b		0.45	0.65				0.65

B

	qRT-PCR	
	SCP2	
181a	1.78	up
181b	1.81	up
181c	1.80	up
181d	1.77	up
181a2*	2.62	up
200a	1.72	up
200b	1.76	up
200c	1.65	up
23a	1.33	up
24	1.12	up
27a	0.75	down
30a	0.63	down
30b	0.64	down
30c	0.65	down
30d	0.64	down
30e	0.62	down
19a	0.52	down
19b	0.46	down
31	1.16	up
21	1.22	up
182	0.73	down
18a	0.54	down
18b	0.56	down
143	1.43	up
374b	1.50	up
374a	1.62	up
877	0.94	down
92a	1.13	up
92b	1.22	up
145	1.22	up
584	0.77	down
151-3p	1.22	up
151-5p	1.30	up
138-1*	1.10	up
34a	0.64	down
365	3.41	up
17	0.85	down
20a	0.78	down
186*	0.21	down
877	1.11	up
513-5p	3.76	up
130a	0.55	down
130b	0.42	down

Fig.4.1: TGFβ-mediated miRNA fold-regulation

Assuming that miRNA effects were being compensated for in our experimental setup by other factors, we decided to assess another tumor suppressive effect of TGFβ after 24 hours. In this shorter experimental setup, other interfering parameters would have less time to compensate for miRNA effects. TGFβ is known to potently arrest cells in G1 of the cell cycle. We tested a few cell lines for the TGFβ-mediated G1 arrest and for this we synchronized cells by serum-starvation and treated cells with TGFβ after returning cells to a 2% FBS medium to allow them to re-enter the cell cycle. We used Smad siRNA as a positive control as Smads were shown to mediate part of TGFβ cytostatic effects. We tested breast cancer cells SUM149PT, SUM159, MCF7 and melanoma cell WM278. SUM149PT was the most responsive cell line to the TGFβ arresting effect (data not shown). We then decided to check the effect of modulating endogenous TGFβ-regulated miRNA on the ability of TGFβ to arrest cells in G1. To this end, we pretransfected cells with 100 nM miRNA modulator oligonucleotides 24 hours prior to TGFβ stimulation and monitored the cellular response to TGFβ stimulation. For TGFβ-induced miRNA, or previously termed up-regulated miRNA, we used antisense oligonucleotides. For TGFβ-inhibited miRNA, or down-regulated miRNA, we used sense oligonucleotides. We monitored the cell cycle by FACS using propidium iodide staining. Shown in **Fig.4.2B**, a subset of TGFβ-regulated miRNA appeared to be involved in TGFβ-mediated cell cycle arrest. Indeed, inhibition of TGFβ-induced or overexpression of TGFβ-inhibited miRNA led to diminished TGFβ cell cycle arresting effects. We observed modulating TGFβ cytostatic effects following miR-30c, miR-30d, miR-30e, miR-31, miR-21, miR-374b modulation. This suggests that these miRNA are involved in TGFβ-mediated G1 arrest. We studied the role of miR-30 downstream in TGFβ-mediated cell cycle arrest in the previous chapter. The observation that miR-31, miR-21 and miR-374b modulation attenuates TGFβ-mediated cell cycle arrest is consistent with recent works showing that miR-31 modulates the cell cycle in lung[451] and thyroid[452]cancer. miR-21 has also been shown to be involved in pediatric cancer stem cell[453] and murine eosinophile[454] proliferation. Less is known about miR-374 but higher expression levels have been reported in leukemia blast cells compared to normal thymocytes[455] and higher expression has been associated with increased overall survival in nonsmall cell lung cancer[456]. This data indicates that a subset of TGFβ-regulated miRNA mediated some of the tumor

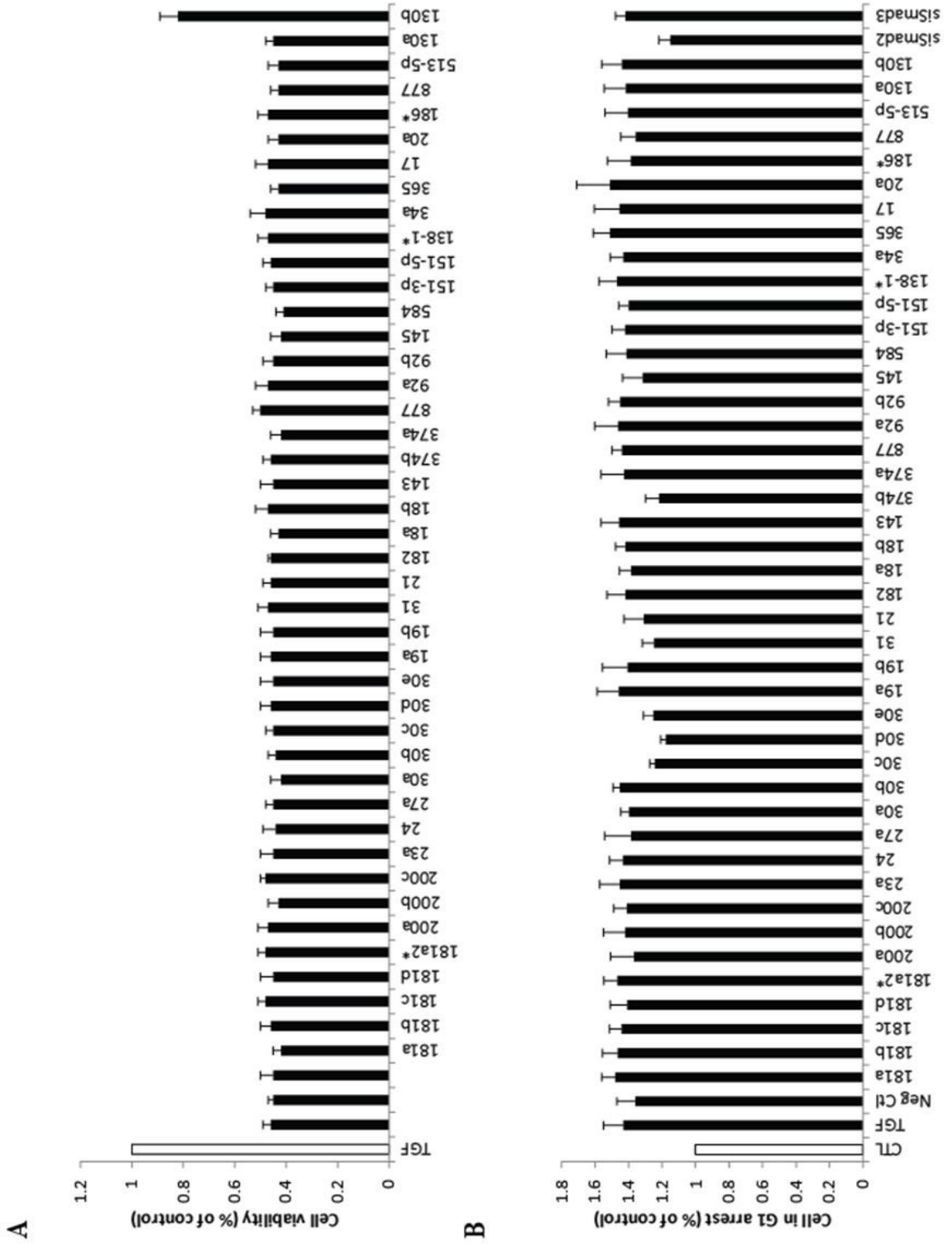
suppressive effects of TGFβ in cancer. This finding is of high therapeutic interest. In order to further understand the contribution of miRNA in TGFβ signalling, we investigated the tumor promoting arm of TGFβ signalling.

4.4.3. TGFβ-regulated microRNA and their role TGFβ-mediated tumor progression

TGFβ acts has been shown to have a potent tumor promoter in late stage breast carcinoma[19]. Numerous miRNA have been associated with tumor progression and metastasis[368,457]. As TGFβ modulated numerous miRNA in our model system, we decided to investigate if TGFβ-modulated miRNA were involved in TGFβ-induced tumor progression. We decided to assess the potential role of TGFβ-regulated miRNA downstream of TGFβ in the mediation of TGFβ pro-migratory effects which are essential for tumor progression to metastasis. For this we assessed the effect of TGFβ on SCP2 cells migration after modulating endogenous TGFβ target miRNA. SCP2 cells were grown to confluence pre-transfected with 100 nM corresponding miRNA modulators 48 h prior to TGFβ stimulation and then allowed to migrate for 24 h in a freshly made wound.

Fig.4.2: Effect of TGFβ-regulated miRNA in TGFβ-mediated tumor suppression

HuH7 and SUM149PT cells were pre-transfected with100 nM miRNA modulators or a negative control sequence (NegCTL) (inhibitors for TGFβ1 up-regulated miRNA or mimics for TGFβ1 down-regulated miRNA) 48 hours prior to being stimulated or not with 100 pM TGFβ1(CTL and TGF). **A**,Cell viability was assessed in HuH7 cells after 48 h of TGFβ1 stimulation and treated conditions were normalized to their non-stimulated counterpart. **B**, Cell cycle distribution was assessed in SUM149PT cells by FACS and accumulation of cells in G1 was observed 24 h after TGFβ stimulation. The transfection conditions are in the same order in both panels.



To determine the pro-migratory effect of TGFβ we normalized the wound area after 24 h to the initial wound size and substracted that closure to the basal closure in the absence of

TGFβ treatment. Disrupting TGFβ-mediated miRNA regulation by transfecting SCP2 cells with synthetic modified oligonucleotides allowed us to assess the functional relevance of individual miRNA by disrupting their regulation by TGFβ. Shown in **Fig.4.3**, TGFβ exerted a smaller pro-migratory response in SCP2 with disrupted levels of miR-181b, miR-181c, miR-181d, miR-24, miR-27a, miR-31, miR-21, miR-92a and miR-130a. These results suggest that these 9 miRNAs are involved in TGFβ-mediated migration as disrupting these TGFβ target miRNA led to decreased TGFβ-mediated induction of migration. These observations are in supported by previous studies associating miR-24 and miR-27a have been associated to migration/invasion[458-460]miR-31 with migration in pancreatic cancer[461,462], miR-21 was associated with hepatoma and cell migration[463,464]. High levels of miR-130a have been associated with better overall survival in glioblastoma[465] and aberrant promoter methylation was shown to decrease miR-130a levels in breast cancer[466]. Taken together these cancer-associated miRNAs appear to be relevant TGFβ targets worth investigating in the context of breast cancer progression to metastasis.

Wound closure (% of initial wound)
0
10
20
30
40
50
60
70
80
90
CTL
TGF
Neg Ctl
181a
181b
181c
181d
181a2*
200a
200b
200c
23a
24
27a
30a
30b
30c
30d
30e
19a
19b
31
21
182
18a
18b
143
374b
374a
877
92a
92b
145
584
151-3p
151-5p
138-1*
34a
365
17
20a
186*
877
513-5p
130a
130b

Fig.4.3: Effect of TGFβ-regulated miRNA on TGFβ-mediated tumor progression

SCP2 cells were pre-transfected with 100 nM miRNA modulators for 48 hours, serum-starved overnight and stimulated or not with 100 pM TGFβ1. SCP2 were allowed to migrate into a wound for 24 hours and TGFβ-mediated wound closure was normalized to non-stimulated wound closure. Wound areas were measured by taking 6 independent photos under an inverted microscope. The arithmetic mean of 4 independent experiments is graphed and error bars are SEM.

4.5. Discussion

Although the role of TGFβ in breast cancer progression has been extensively studied, our understanding of the role of miRNA in cancer has only been developed this past decade. Since the first association of miRNA and cancer in 2002[270], many miRNA have been associated with diseases and cancer. Few miRNAs have been associated directly to breast cancer progression.

The explosion of interest in miRNAs over the past two years necessitates effective tools for detecting their presence, quantification, and functional analysis. High-throughput miRNA microarrays have facilitated miRNA expression profiling. These methods are far superior to existing low through-put techniques such as time-consuming qRT-PCR which are essential for validation of microarray data.

Locked nucleic acid (LNA) modification improved oligonucleotide thermostability and increased specificity, thus enabling miRNAs with single nucleotide differences within a miRNA family such as miR-181 or miR-30 to be discriminated, an important consideration as sequence-related family members may be involved in different physiological functions[467].

This study identified several miRNAs which are under TGFβ control and that seem to mediate in part the TGFβ tumor suppressive and tumor promoting effects in breast cancer cell lines. Supported by recent studies, our work sheds light on major contribution of miRNA downstream of TGFβ. The association of aberrant TGFβ-mediated miRNA regulation of expression with progression of tumorigenesis and the functional analysis of specific miRNAs illustrate the feasibility of using miRNAs as targets of therapeutic intervention. Anti-miRNA 2-*O*-methyl or LNA oligonucleotides used to inactivate oncomirs such as miR-181[401] MicroRNA correction therapies can be combined with other therapies[468]. Suppression of endogenous miRNA activity and its downstream effect on

mRNA expression achievable both *in vitro* and *in vivo* [469-472]. The effects of target miRNA knockdown on cell morphology and function can be determined using standard assays for processes such as cell proliferation, migration, invasion, and angiogenesis. Characterizing miRNA functions in vivo can also be carried out with intravenous injections of antagomirs[271]. Recent developments in the miRNA inhibition described novel techniques to manipulate miRNA expression *in vivo* by expressing decoy miRNA targets via lentiviral vectors[473].

The ability to obtain mRNA expression profiles from human breast tumors has led to remarkable insight and knowledge regarding the characterization of different lineages and tumour clustering. Elucidation of the molecular mechanisms involved in breast cancer has been the subject of extensive research in recent years, but major challenges persist in the management of breast cancer patients including unpredictable response and development of resistance to adjuvant therapies. The triple negative breast cancer subtype is one for which few therapeutic strategies have been developed so far. It has been shown that distinct patterns of miRNA expression are observed in different breast cancer lineages, reflecting the mechanisms of transformation, and further supporting the idea that miRNA expression patterns encode the developmental carcinogenetic history. Our previous work on miR-584[367], miR-181[401] and miR-30 support the idea that targeting endogenous miRNA with LNA-based miRNA modulators can be effective in triple negative breast cancer cells.

Of the 550 existing miRNA at the time of our microarray, we isolated 43 miRNA with different levels of TGFβ regulation and confirmed their regulation by qRT-PCR. We further characterized their role in mediating TGFβ-induced cell cycle arrest and cell migration in triple negative breast cancer cells. Interestingly, little is known about miR-374 which we have seen to be involved in TGFβ-mediated cell cycle arrest. We also report a role for miR-130 in TGFβ-induced breast cancer cell migration. These findings open the path to further research on potentially novel triple negative breast cancer miRNA correction therapies.

Chapter 5

General Discussion

Genomic and proteomic approaches have generated results that changed our view on cancer. The disease was initially believed to result from the alteration of only several genes, but cancer has been shown to be genetically more complex. Tumor cells frequently have an array of mutations, and each tumor mass contains hundreds of cancer cells with distinct sets of mutations[474,475]. This observation led to a shift in cancer therapies to accommodate these important new findings and to produce safer and more effective remedies. The prevailing forms of therapy are frequently too toxic or even prove inadequate because of chemoresistance. These classic treatments are gradually being replaced by more targeted therapies that target known oncogene. Increasingly, targeted therapies are prescribed not only based on the cancer histotype but [illegible]etype. Therapies originally developed for a specific tumor type can [illegible]ectively be used in another hi[illegible]e[illegible]ano[illegible]e as long as the underlying mutation are similar[476]. At this point in time, many targeted therapies still have insufficient

effectiveness and are used in combination. This shows that cancer is the result of many genetic and epigenetic alterations and requires a complex interference with multiple cancer pathways. MicroRNAs have been shown to be able to target hundreds[261] of target genes in multiple pathways and as such represent a highly promising. The strongest rationale for exploring the potential of therapeutic use of miRNAs lies in the ability of miRNA to regulate multiple oncogenes and oncogenic pathways that are commonly deregulated[477].

The first published miRNA report occurred in 1993 and resulted from genetic screening in worms. The worm miRNA was the only known small RNA until a second miRNA was discovered in 1999. Until 2001, miRNAs appeared to be a worm-specific oddity, when the cloning and sequencing of over 100 human and murine miRNAs was published[265]. Since then there has been an exponential increase in the number of miRNA-related publications, in particular regarding their contributions to cancer development in the recent years.

With abundant profiling and gain-and-loss of function studies, the major role of miRNA in many different diseases in both animals and humans is widely appreciated[478]. The increasing awareness of the importance of miRNAs has prompted intense effort within biomedical research community. Several laboratories, including industry (http://www.mirnatherapeutics.com), have shown that restoring miRNA function can reduce tumor growth *in vitro* and *in vivo*. A well-documented example is the tumor-suppressor activity of miR-34 which inhibits tumor growth in murine models of non-small cell lung cancer, prostate and liver carcinoma, and B-cell lymphoma[479-482]. The industry has established proof-of-concept for the systemic delivery of a synthetic miRNA in murine cancer models and is bringing lead candidates to clinical trials[483]. As discussed in this thesis, miRNAs act as "master switches" of the transcriptome, extensively regulating gene products and coordinating various pathways. Recent studies have shown that miRNAs influence the epigenome; miR-29 was shown to influence tumor-related gene promoter methylation and to influence disease outcome[484] by targeting DNA methyltransferase 3A and 3 B[485,486]. Many miRNA-regulated genes include conventional oncogenes and tumor suppressors, many of which are individually pursued as drug targets

by the pharmaceutical and biotechnology industry. For this reason miRNA therapeutics may have far superior activity by co-targeting multiple cancer-related genes. As mis-regulation of miRNAs is often an early event in tumorigenesis, miRNA-based therapeutics, which replace missing miRNAs, may prove to be the most relevant therapy. Experimental evidence demonstrate that correction of specific miRNA alterations using miRNA sense oligonucleotides or mimics or antisense oligonucleotides or antagomirs can normalize gene regulatory networks and signaling pathways thereby reversing cancerous phenotype in cells. Foreseeably the change of a single miRNA expression in the miRNA/mRNA network can trigger a chain reaction; when the changes reach the threshold, the cells may change the biological behavior. The idea of miRNA modulating thus possesses great potential both as a novel class of therapeutic targets and as a powerful intervention tool[487,488].

There are essentially two therapeutic miRNA-related strategies.

(1) If the diseased tissue is afflicted with a gain-of-function miRNAs or oncogenic miRNAs, the oncomiR can be inhibited with antagonists antisense oligonucleotides such as antagomiRs. For instance, miR-208 is a intronic miRNA that nearly only expressed in the heart and encoded in the alpha-myosin heavy chain (α-MHC) gene and stimulates cardiomyocyte hypertrophy, fibrosis and β-MHC expression in response to stress. Knock-out mice deficient in miR-208 were resistant to fibrosis and cardiomyocyte hypertrophy suggesting that an antagonistic approach to miR-208 could be of therapeutic value in chronic heart disease[489]. The phenomenon of oncogene addiction reveals that despite the multistep nature of tumorigenesis, bottlenecks exist and the targeting of certain single oncogenes can be of therapeutic interest[490,491]. The possibility of oncomiR addiction was a promising idea until miR-21 oncomiR addiction was demonstrated in pre-B-cell lymphoma[492].

(2) If loss-of-function of miRNAs lead to disease, in the case of tumor suppressor miRNAs, miRNA activity can be restored by using miRNA mimics. Several

important observations support the miRNA replacement therapy: (i) most differentially expressed miRNAs are suppressed in tumor versus normal tissue, so the probability for miRNAs being a tumor suppressors miRNA is greater than the it being an oncomiR[493] and (ii) inhibition of miRNA processing itself induces oncogenic transformations which promotes tumorigenesis, suggesting once again that the tumor suppressive role of miRNAs dominates the oncogenic role[494]. Importantly, since miRNA therapeutic mimics have the same sequence as the depleted endogenous naturally occurring miRNA, they are expected to share the same set of target mRNAs as the natural miRNA. The therapeutic miRNA activity can be enhanced through several molecular backbone chemical alterations and with use of an adjuvant which protects the oligonucleotide from biodegradation. The steps prevent rapid kidney-mediated clearance and improve the therapeutic miRNA uptake by target cells. These adjuvants, referred to as "delivery technologies", allow for a more practical drug administration route. The administration route is systemic delivery since it disseminates the therapeutic more effectively[495]. When possible local administration can be equally effective. For example, the expression of miR-29b is reduced in non-small-cell lung cancer and systemic delivery using a cationic lipoplex in a murine model led to reduced tumor growth[496]. The disadvantages however of this miRNA mimic therapies are that oligonucleotides a short half-life and therefore transient effects thus requiring repeated supplementation. Also another important determinant in drug development is safety, so the tissue-specific miRNAs will be preferred, especially for the more chronic indications requiring repeated therapeutic treatments. Recently efforts have produced RNA polymerase II expression vector including miRNA flanking sequences[497] used in *in vitro* and *in vivo* studies[498-500]. However, these studies were limited on miRNA-expressed cells in vitro and in xenograft models. Targeted direct delivery of miRNA-mimic oligos or vector-based miRNA expression to specific tumors or organs remains to be addressed.

It is in this context that this current thesis sheds light on several promising miRNAs with high therapeutic potential in TNBC for which little to no therapies are currently available. This thesis supports the current enthusiasm for miRNA-based therapies in the treatmet of diseases including TNBC. The miR-181 is often overexpressed in cancer. We have demonstrated in Chapter 2[401] that targeting endogenous TGFβ-induced miR-181 using 2'O-methyl-modified antisense oligonucleotides *in vitro* did not affect the tumor suppressive effects of TGFβ therefore would potentially not interfere with the stroma while decreasing TGFβ prometastatic effect. Indeed, metastatic triple negative breast cancer cells treated with anti-miR-181 oligonucleotides were less migrative and invasive following TGFβ stimulation. This finding is of interest because in the context of aggressive metastatic breast cancer, the cancer cells produce TGFβ into the environment and this secreted TGFβ has a profound autocrine effect favoring tumor progression to metastasis. Our data suggest that treating metastatic triple negative breast cancer could reduce tumor progression and as such could be used in combination therapies. Our second study identified miR-30 as being a TGFβ-inhibited miRNA of interest. This miR-30 family is often deregulated in cancer. Interestingly, miR-30 down-regulation appeared necessary for TGFβ tumor suppressive effects both for cell cycle arrest and apoptosis. We identified potential miR-30 downstream target genes which are modulated by miR-30 in triple negative breast cancer. This second study enhances our understanding of the TGFβ biological responses in triple negative breast cancer cells and can lead to new therapeutic avenues. The final chapter of this thesis investigates 43 TGFβ-regulated miRNA including the four miR-181 family members and the five miR-30 family members. Several of the identified miRNA of interest have been associated with cancers including breast cancer which validates our strategy. Importantly, we characterize a subset of new TGFβ-regulated miRNA of interest downstream of TGFβ signalling. We identify miR-31, miR-21 and miR-374b downstream of TGFβ-induced cell cycle arrest. Interestingly, an important high throughput functional study using robotics was carried out by Zhang *et al*.[501] and tested the effect of 904 miRNAs for migration in hepatocarcinoma, cervical carcinoma and osteosarcoma cells using printer-assisted miRNA spotting on a chip. Zhang deemed the "study of cell motility in parallel [to be] a formidably challenging

task" and devised a high throughput method. We manually tested the effect of miRNA modulation with both sense and antisense modulators for 43 independent miRNA.

We also identify miR-24, miR-27a, miR-31, miR-21, miR-92a and miR-130a as being miRNA of interest downstream of TGFβ promigratory response in triple negative breast cancer cells. Importantly, little is known about the miR-374b and this miRNA had not yet been characterized as being a TGFβ downstream target gene. Unlike the poorly characterized TGFβ-inhibited miR-374b, the TGFβ-induced miR-374a has been characterized as a metastamiR in breast cancer[502] and was first identified in embryonic stem cell[503]. We observe an involvement of miR-130a in TGFβ-induced breast cancer cell migration. Taken together, this thesis work illustrates several examples of promising beneficial effects of miRNA modulation in the context of TNBC using validated modified synthetic nucleic acid oligomers. These exciting priliminary *in vitro* findings need to be confirmed in an *in vivo* setting.

As described in the introduction of this present thesis and detailed in Humbert *et al.* 2010[14], many efforts to target TGFβ signaling in therapy have been developed including TβRI kinase inhibitors, TGFβ-specific neutralizing antibodies and antisense oligonucleotides. These strategies are not successful to date as TGFβ signaling is context-dependent. In the early stage of breast carcinoma TGFβ can inhibit tumor growth and in later stage promote metastasis. Cancer cells phenotypic diversity will lead to a differential response to TGFβ signaling within the same tumor. For treatment of triple negative breast cancer patients with advanced stage tumors, we suggest to use miRNA correction therapies in combination with currently available therapies as a means of improvement.

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