Role of Scleraxis in regulating Snai1 gene expression and its effect on Epithelial –Mesenchymal Transition

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❖ **Abstract:**

Epithelial-mesenchymal transition (EMT) is a process in which epithelial cells lose their ability for cell to cell adhesion, causing a pheno-conversion of epithelial cells into the migratory phenotype of mesenchymal cells. EMT is induced in part by Snai1 expression which directly represses E-Cadherin transcription. E-Cadherin plays an integral role in forming adherens junctions that bind cells together. The EMT process plays an essential role in physiological trans-differentiation of cells during development and contributes to pathological responses and cancer progression.

Snai1 is a zinc-finger transcription factor that triggers EMT during embryonic development as well as in pathological processes such as tumors or fibrosis. Scleraxis is a transcription factor that significantly increases the expression of important extracellular matrix genes such as type I collagen and the contractile protein α-smooth muscle actin (α -SMA), which are key players of the fibrotic process. Our published data suggests that Scleraxis overexpression in the adenocarcinomic human alveolar basal epithelial cells (A549 cells) down-regulates some epithelial markers while up-regulating mesenchymal markers, thus contributing to EMT. Here we show that Scleraxis regulates the transcriptional activity of the *Snai1* gene through direct binding to E-box sequences within its promoter. Additionally, we show that Scleraxis enhances EMT progression

mediated by its interaction with the Snai1 gene promoter. These findings suggest that Scleraxis –Snai1 axis is a potential therapeutic candidate that could be targeted in pathological processes where EMT is elevated.

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"Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time." – Thomas A. Edison

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AF	Atrial Fibrillation
AV	Atrioventricular
bHLH	Basic Helix-Loop-Helix
CF's	Cardiac Fibroblasts
ChIP	Chromatin Immunoprecipitation
COL1A2	Human Collagen 1α2
CtBP	Carboxy-terminal binding protein
CTGF	Connecting Tissue Growth Factor
DNMTS	DNA Methyltransferase
E	Embryonic Day
E-Box	DNA binding site
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
Egr 1	Early Growth Response 1
EMSA	Electrophoretic Mobility Shif Assay
EMT	Epithelial- Mesenchymal Transition
EMT-TFs	EMT- inducing transcription factors
EndMT	Endothelial-to-mesenchymal Transition
EPDCs	Epicardial Derived Cells
FGF	Fibroblast Growth Factor
GFP	Green Fluorescent Protein
HCM	Hypertrophic Cardiomyopathy
HGF	Hepatocyte Growth Factor
HMGA2	High Mobility Group A2
HMTs	Histone Methyltransferase
ILK	Integrin-linked Kinase
LEF1	Lymphoid Enhancer-binding Factor

MAPK	Mitogen- activated protein kinase
MET	Mesenchymal-epithelial Transition
MFB's	Myofibroblasts
MI	Myocardial Infarction
MMP's	Matrix Metalloproteinases
NF κB	Nuclear Factor κB
PDL	Periodontal Ligament
PEO	Proepicardial Organ
PI3K	Phosphatidylinositol 3-kinase
PPAR*y*	Peroxisome Proliferator-activated Receptor *y*
RT-PCR	Real Time Polymerase Chain Reaction
Slug	Snail2
TGF-β1	Transforming Growth Factor-β1
TβRII	TGF-β type II receptors
Zeb1 or 2	Zinc Finger E-box Binding Homeobox 1 or 2
ZO-1	Zona Occludins
α-SMA	α-Smooth Muscle Actin

❖ Introduction

I. Epithelial – Mesenchymal Transition

Initially, the process was described as "epithelial-mesenchymal transformation" by Elizabeth Hay when using a chick primitive streak formation model [1]. Then the term 'transformation' was replaced with 'transition' to emphasize the transient nature of this process which is distinct from the irreversibility of neoplastic transformation [2]. This means that the EMT process involves a phenotypic plasticity by which mesenchymal cells could also convert back to their epithelial derivatives in a process known as mesenchymal-epithelial transition (MET). The best studied example of a MET event is in the formation of the nephron epithelium in the developing kidney [3].

Under normal conditions, epithelial cells form layers that are tightly connected laterally by specialized junctions, including adherens junctions, desmosomes, tight junctions and gap junctions. In particular, adherens junctions have a critical role in constructing lateral cell adhesion epithelium that is anchored to the basement membrane to ensure a restricted migration of cells laterally along the basal surface, thereby maintaining their positioning and generating a barrier to cells penetrating into the underlying ECM [4].

When the EMT process is initiated, epithelial cells lose their basal –apical polarity and cell-cell adhesion, and this is typically accompanied by downregulation of adherens junction proteins, such as E-Cadherin, β-catenin, γ-catenin, and p120 catenin, and tight junctions become disrupted through downregulation or delocalization of tight junction proteins such as ZO-1, occludin, and claudins. These changes allow cells undergoing EMT to develop mesenchymal features, like spindle-shaped morphology, reorganization of actin stress fibers and adoption of a front-back end polarity. Cells also establish a migratory invasive capacity, elevated resistance to apoptosis

as well as an increased expression of mesenchymal marker proteins such as N-cadherin, vimentin, and fibronectin [5]. Eventually, this process results in degradation of the basement membrane and migration of the mesenchymal cells away from the epithelial layer [2].

1. EMT Subtypes

In 2007, it was proposed that EMT can be classified into three biological subtypes based on their biological context; 'Type1 EMT' is associated with implantation, embryo formation and organ development; this includes its capability of generating mesenchymal cells that may have the potential to subsequently undergo MET [6]. 'Type 2 EMT' is associated with wound healing, tissue regeneration and organ fibrosis. This type is induced following trauma or inflammation injury that initiates a repair response mechanism and when persists it can lead to fibrosis [2]. 'Type3 EMT' contributes to cancer progression through the generation of invasive neoplastic cells that have been previously undergone genetic and epigenetic changes which alter the normal mechanism of cell differentiation.

1.1. EMT Role in Development

EMT is associated with almost all forms of development. It can be broadly involved in different tissue remodeling events such as mesoderm formation, cardiac and neural crest development and secondary palate formation [7]. Initially, epithelial cells start to develop about 8 days post fertilization. During early embryonic development, mesoderm layers are initially formed by the invagination of epithelial cells through the basement membrane, by which they undergo drastic morphological changes where they differentiate into mesenchymal cells and migrate along the ectodermal narrow space [8]. At this point, these ectoderm-derived cells gain

the ability to penetrate the ECM, which marks the final stage in EMT program during gastrulation.

Another critical event requiring EMT during embryogenesis is neural crest formation [9]. The neural crest develops in between the neural plate and the epidermal ectoderm, by which neural crest cells lose N-cadherin-mediated cell-cell adhesion and become excluded from the neural epithelium, and basal lamina become disrupted immediately to facilitate neural crest cell migration in the cranial regions of avian and mouse embryos [10].

As neural crest cells migrate from the neural epithelium, a constant up-regulation of genes required for the mesenchymal phenotype is expressed. Later, a significant amounts of fibronectin and hyaluronan are expressed along the path of cell migration, to facilitate a controlled migration of cells to the appropriate destinations [11].

It is important to emphasize the role of EMT in the developing heart. Pericardial progenitor cells arise from splanchnic mesoderm via MET, then assemble to create a transitory body of cells called the 'proepicardial organ.' As they lay adjacent to the sinus venosus, they undergo EMT and generate a mesenchymal population of epicardial-derived cells (EPDCs) that migrate within the pericardium and attach to the myocardial surface, then proliferate and flatten to cover the embryonic heart as a continuous epicardial sheet[12-14]. Some of this population of EPDCs occupy the ECM region between the epicardium and myocardium named the 'subepicardial space', while others migrate further to invade the myocardium [15]. A strong body of evidence indicates that EMT-derived EPDCs are the primary source of coronary endothelial cells, coronary vascular smooth muscle cells and cardiac fibroblasts [16, 17].

Additionally, detailed molecular studies have identified the role of EMT in cardiac valve formation. The heart valves are originally formed from a precursor structure, the endocardial cushions, soon after the primitive linear heart tube begins to loop. Initially, cells from the myocardium secrete a large amount of ECM to create more specialized layers of endocardial cushions that are distinct from myocardium [18]. Later, signals secreted from atrioventricular (AV) myocardium induce EMT in the AV endocardial cell layer by which AV endocardial cells lose their intercellular attachments and penetrate the endocardial cushion, thus increasing the number of mesenchymal cells that fill the cushion space to be involved in developing the cardiac septa and valves[19-21].

Another example to which the EMT program contributes is in palatogenesis. During secondary palate development, the palatal shelves tends to fuse in the midline. As these shelves approach each other from the opposite sides of the oral cavity, epithelial cells covering the tip of each shelf and are combined. Soon after fusion is complete, these medial epithelial cells undergo EMT and become part of the mesenchymal compartment in the palate [15].

1.2. EMT Role in Fibrosis

Organ fibrosis occurs in multiple epithelial tissues, and is mediated by inflammatory cells and fibroblasts that accumulate and secrete excessive inflammatory signals as well as components of a complex ECM that compromises organ function and leads to its failure. Many diseases can develop as a result of fibrosis occurring in heart, kidney, liver, lung, and intestine [22-24].

Multiple cell tracing studies using transgenic reporter mice have shown that a large number of the myofibroblasts that are involved in interstitial kidney fibrosis arise from the conversion of

epithelial cells through EMT during renal fibrogenesis [25]. Moreover, lineage-tracing experiments in transgenic adult mice indicate that hepatocytes undergo EMT during CCl_4-induced liver fibrosis [23]. Other studies have shown that hepatocytes that are derived from cirrhotic liver display some characteristics, such as increased vimentin and type 1 collagen expression, which are consistent morphological features with EMT, and would have an implication for the progression to hepatocellular carcinoma [20]. *In vivo* studies reveal the role of alveolar epithelial cells as progenitors for fibroblasts, as they undergo EMT during pulmonary fibrosis [24].

Interestingly, lineage tracing analysis using the pressure overload mouse model showed that cardiac fibrosis is associated with the emergence of fibroblasts that originate from endothelial cells, suggesting that the process of EndMT contributes to cardiac fibrosis [22].

Supporting evidence for EMT has arisen in other models of cardiac fibrosis, including hypertrophic cardiomyopathy (HCM), a disorder characterized by myocyte enlargement, fibrosis, and impaired ventricular relaxation [26]. In this disorder, non-myocyte cells exhibit EMT-like characteristics by which they become activated and proliferate to produce the pathologic remodeling in HCM. Another study suggests that diabetes mellitus-induced cardiac fibrosis is associated with the emergence of fibroblasts from endothelial cells and this EndMT process is stimulated by Endothelin-1 [21].

Conversely, a recent study by the Molkentin group, also using lineage tracing experiments, showed that myofibroblasts in mouse hearts post-MI are predominantly derived from tissue-resident fibroblasts rather than from endothelial cells or other proposed cell sources [27].

Together, this shows that the origin of fibroblasts that contributes to cardiac fibrosis remains unclear, and it is still controversial whether the EMT process can be correlated to a specific cardiac injury. But it is clear that EMT is of key importance in the fibrotic remodeling of certain tissue injuries.

1.3. EMT Role in Cancer Progression

Invasion of the surrounding tissues and metastasis to distant organs are the initial features of malignancy which have been extensively correlated with EMT. Initially within metastasis, tumor cells dissociate from the epithelial layer, gain mobility and penetrate through the basement membrane to invade adjacent tissues, penetrate the endothelial barrier and migrate through the bloodstream, then extravasate at a distant site. Eventually, metastatic cells grow at the distant site with stimulation of neoangiogenesis [28]. As EMT provides a mechanism for tumor cells to metastasize and leave the primary tumor to invade into blood vessels, it contributes to tumor progression. Thus, from a clinical aspect, increased expression of positive regulators of EMT in cancer cells are correlated with poor patient outcomes and tumor aggressiveness [29].

About 80% of malignant tumors are from an epithelial origin, among them, features of EMT have been observed in tumors of breast, esophagus, colon, ovary and others [30-34]. In such tumors, cells exhibit a motile and invasive phenotype parallel to the changes observed during developmental EMT [35, 36]. This supports the well-known hypothesis that developmental programs are reactivated during disease or tumor progression, thus many EMT regulators in development become inappropriately expressed in human cancer and are correlated with EMT features. Many inducers of EMT in cancer cell lines have been identified including TGF-β, Wnt, Snail/Slug, and Twist , which are also critical during developmental EMT program[26, 37-42].

2. EMT Signaling Pathways

TGF-β is one of the key players that induce EMT, along with various humoral factors including epidermal growth factor (EGF), fibroblast growth factor (FGF) , hepatocyte growth factor (HGF), Wnt and ECM components [30].

2.1. TGF-β Signaling in EMT

TGF-β signaling acts through various intracellular messengers to induce EMT. Typically, EMT is activated by the TGF-β superfamily of ligands, including but not restricted to the three isoforms of TGF-β (TGF-β1, 2, and 3). Many studies have shown that TGF-β1 signaling contributes to EMT-associated cancer and fibrosis [31]. In contrast, TGF-β2 primarily controls EndMT during heart development and TGF-β3 mediates EMT in the developing palate [32, 43].

TGF-β signaling occurs through formation of a heterotetramic receptor complex combining type I and type II TGF-β serine-threonine kinase receptors (TGF-βRI and TGF-βRII) [33, 34]. Upon ligand binding, TGF-βRII trans-phosphorylates TGF-βRI, facilitating its kinase activity, where they can act either on Smad dependent or Smad-independent pathways. Differential signaling is enabled due to the same ligand binding to a different receptor combination. Also, this combination of receptors enables differential ligand binding, thus responding to multiple isoforms of the TGF-β superfamily [34 ,44].

2.1.1. Smad-Dependent Pathway

Phosphorylation of TGF-βRI by TGF-βRII recruits Smad2 and 3 where they bind to form a complex with Smad4, then this heteroduplex translocate to the nucleus [45]. Once inside the nucleus, Smad complexes bind regulatory elements and induce the transcription of key genes associated with EMT.

TGF-β signaling through Smads can enhance lymphoid enhancer-binding factor (LEF1) expression, which triggers the β-catenin-dependent pathway that is responsible for the complete loss of E-Cadherin and transformation to the mesenchymal phenotype, thus leading to EMT [46].

Additionally, in TGF-β - Smad signaling, EMT is induced when the Smad complex binds directly to the *Snai1* gene promoter and forms a complex to downregulate the expression of genes encoding E-Cadherin and occludin [47, 48]. Other factors are influenced by this signaling such as Zeb1 and Zeb2 transcription factors which also contribute to E-Cadherin repression. On other hand, TGF-β-associated Smad complexes are known to increase N-cadherin, fibronectin and α-SMA expression [44].

2.1.2. Smad-Independent Pathway

The contribution of TGF-β in EMT is not limited to Smad signaling. TGF-β can also activate Ras/MAPK, PI3K/Akt , Rho/Rac, integrin-linked kinase (ILK), and Wnt/β-catenin pathways to trigger EMT [49, 50]. In general, most downstream mediators of receptor tyrosine kinases, such as MAPK, p13K and Rho GTPases work with TGF-β to induce EMT. Together, the TGF-β cytokines are considered the main inducers of EMT through diverse cellular mechanisms.

2.2. Wnt/β-Catenin Pathway

In the absence of Wnt signals, β-catenin is associated with E-Cadherin at the adherens junction. Usually the non-sequestered cytoplasmic β-catenin is rapidly phosphorylated by GSK-3β and degraded in the cytoplasm [51].

However, when Wnt glycoprotein ligand binds to the frizzled receptor, GSK-3β is unable to phosphorylate β-catenin, thus the degradation of β-catenin is inhibited leading to its

accumulation in the cytoplasm followed by translocation to the nucleus and binding to LCF/TEF transcription factors which leads to modulation of the expression of genes involved in EMT induction [51]. Additionally, Wnt signaling can be induced through E-Cadherin down-regulation, by which β-catenin levels increase in cytoplasm [43].

2.3. Notch Signaling Pathway

The Notch family consists of transmembrane receptor proteins. The Notch ligand binds to its receptor then initiates downstream signalling pathway. Notch cooperates with TGFβ to induce EMT. This pathway mainly maintains the balance between apoptosis and proliferation in many organs. Therefore, any dysregulation of this pathway would trigger tumor progression through activation of Notch that leads to Snai1 upregulation, repression of E-cadherin and eventually induction of EMT [52].

2.4. Hedgehog Pathway

This pathway is highly correlated to tumorigenesis. There is robust evidence that Hedgehog signaling leads to upregulation of Snai1, triggering the expression of the downstream transcription factor Gli-1 and causing an enhanced metastasis in the prostate cancer mouse model. Targeting this pathway with the inhibition of cyclopamine inhibits tumor progression. This indicates that this pathway contributes with EMT during tumor progression [52]. Furthermore, Hedgehog upregulates TGFβ secretion to promote cancer cell motility and invasiveness [53].

3. EMT-Inducing Transcription Factors

EMT is associated with changes in gene expression that contribute to repression of the epithelial phenotype and induction of the mesenchymal phenotype. This is mediated by the

classical EMT-inducing transcription factors (EMT-TFs) [54]. These transcription factors have a distinct expression profile, thus, their contribution to EMT is tissue-specific. Moreover, they can regulate the expression of each other and functionally cooperate at target genes.

A phenomenon known as the 'cadherin switch' is the hallmark of EMT, characterized by down-regulation of E-Cadherin and up-regulation of N-Cadherin. Thus, E-Cadherin, the key marker of epithelial cells, is targeted by EMT-TFs, contributing to the down-regulation of the genes that are essential for the maintenance of tight junctions or desmosomes in the epithelial cells, including those encoding claudin, occludin, desmoplakin and plakophilin. On the other hand, EMT-TFs activate the expression of mesenchyme-associated genes such as N-Cadherin, fibronectin and vimentin [30].

EMT-TFs can be divided into two groups based on the mechanism of E-Cadherin suppression. Snai1, Slug, Zeb1, Zeb2, E47, KLF8, and Brachyury directly bind to the E-Cadherin gene promoter and suppress its expression, whereas Twist1, FOXC2, Goosecoid, E2-2, Six1, and Prrx1 trigger EMT without direct interacting with the E-Cadherin gene promoter [54].

More specifically, EMT is orchestrated by a restricted number of transcription factors, mainly the Snai1, Twist1, and Zeb superfamilies (EMT-TFs).

3.1. Zeb Family Factors

This family consists of Zeb1 and Zeb2 members, which are characterized by the presence of zinc-finger clusters at each terminus. These factors interact with DNA through binding of the two zinc-finger domains to DNA binding sites (E-boxes) [55]. Zebs have a dual transcriptional activity: they function as both transcriptional repressors and activators, thereby

repressing some epithelial junction and polarity genes and activating mesenchymal genes that define the EMT phenotype [56].

Zeb expression is induced in response to TGF-β1 and Wnt proteins through activation of RAS– MAPK signalling pathway [56]. Also, Zeb1 transcription is controlled by Snail1 at multiple levels and its expression is induced by Snail1 cooperatively with Twist1. [57]

3.2. Twist

Twist is one of the most important factors that regulate EMT. This highly conserved basic helix-loop-helix factor has a crucial role for proper gastrulation and mesoderm formation [58]. Additionally, it plays a significant role in tumor progression. Exogenous overexpression of Twist1 increases the invasive and metastatic abilities of human cancer cells by promoting downregulation of E-Cadherin and inducing EMT [39, 59].

Although Twist1 proteins are commonly recognized as indirect repressors of E-Cadherin, they can also bind directly to E-boxes 2 and 3 present within the E-Cadherin gene promoter to repress its expression [60].

3.3. Snail

3.3.1. Snail Superfamily of Zinc-Finger Transcription Factors

Snail family members encode transcription factors of the zinc-finger type including Snai1, Snail2 (Slug) and Snail3 (Smuc) [61]. They are similarly organized, being composed of a highly conserved carboxy-terminal region that contains from four to six zinc fingers, and a much more divergent amino-terminal region. The zinc fingers function as sequence-specific DNA-binding motifs and are structurally composed of two β-strands followed by an α-helix, the

amino-terminal part of which binds to the major groove of the DNA. The two conserved cysteines and histidines (C2H2) coordinate the zinc ion [61].

Snail family is associated with many processes that involve cell movements, both during embryonic development and in the acquisition of invasive and migratory properties during tumor progression. Various members of this family have also been implicated in neural differentiation, cell division and cell survival [61].

Different transfection tests using random or selected promoters have shown that the Snail-related genes contain one E-box or more in their promoters [62, 63]. These consensus binding sites are similar to those of bHLH transcription factors, indicating that Snail proteins might compete with them for the same binding sequences [64, 63].

3.3.2. Snai1 Characteristics and Regulation

Snai1 is the main regulator in both developmental and pathological EMT. Structurally, the Snai1 gene promoter consists of several conserved regulatory elements including AP1 and AP4 sites, LEF1 binding sites, Smad-binding elements and E-boxes [55]. The Snai1 protein has two functional conserved domains which are: a C-terminal DNA-binding domain that has a high affinity for the CACCTG sequence [62] and an N-terminal regulatory domain or called SNAG domain which is necessary for transcriptional repression [65].

Upon binding to the E-box, the repressive role of Snai1 depends on either its zinc finger region or on its two different motifs that are found in the amino-terminal region [66].Thus, the repressor activity of Snai1 proteins has been identified through different mechanisms: CtBP (carboxy-terminal binding protein) co-repression or SNAG domain acting alone, or both in conjunction [67]

3.3.2.1. Snai1 Regulation

Snai1 gene transcription is regulated by various signaling pathways that are involved in EMT in several cell systems, including PI3 kinase (PI3K), ILK and GSK-3β and NF κB [48], [68]. The Wnt signaling pathway is another example by which Snai1 is regulated. A key member of the Wnt signaling cascade is β-catenin. Upon Wnt activation, β-catenin accumulate and translocate to the nucleus where it transactivates multiple downstream regulators that induce EMT. Immunoprecipitation studies show that Snai1 functionally interacts with β-catenin to increase Wnt-dependent target gene expression [69, 70].

Additionally, in the TGF-β induced EMT pathway, Smad3/4 form a complex with the high mobility group A2 (HMGA2) and cooperatively bind to the Snai1 gene promoter to activate its transcription [71]. Another example of how Snai1 expression being up- regulated is by hepatocyte growth factor (HGF) which is mediated via the MAPK/Egr-1 pathway. Both Snai1 and early growth response 1 (Egr1) proteins have a critical role in HGF-induced cell scattering, migration and invasion [72].

Interestingly, Snai1 is subjected to auto-regulation at the transcriptional level. Snai1 protein acts as a negative regulator by binding to the functional CACCTG E-box sequence within the Snai1 gene promoter, creating a negative feedback loop to repress its own gene expression [73]. This self-inhibitory mechanism ensures a precise control of Snai1 activity which could be critical during embryonic development.

3.3.2.2. Snai1 Repressor Activity

Snai1 binds directly to the E-Cadherin gene promoter through its E-box sequence and represses E-Cadherin transcription. This mechanism was identified through various studies

involving the overexpression of Snai1 in different epithelial cells, and leads to a dramatic conversion towards a fibroblastic phenotype while E-Cadherin expression is lost, and tumorigenic and invasive/migratory properties are acquired. Moreover, Snai1 expression is observed in E-Cadherin-deficient murine and human carcinoma cell lines and in invasive regions of carcinomas, but is absent from well differentiated non-invasive mouse and human carcinomas [66].

Snai1 activity is not restricted to E-Cadherin downregulation. Snai1 is involved in both the direct transcriptional repression of genes, such as E-Cadherin, occludin and claudin-1and post-transcriptional events, including downregulation of ZO-1(zona occludins)[74]. Collectively, this supports the idea that Snai1 is a transcription factor that represses various intracellular adhesiveness elements [75].

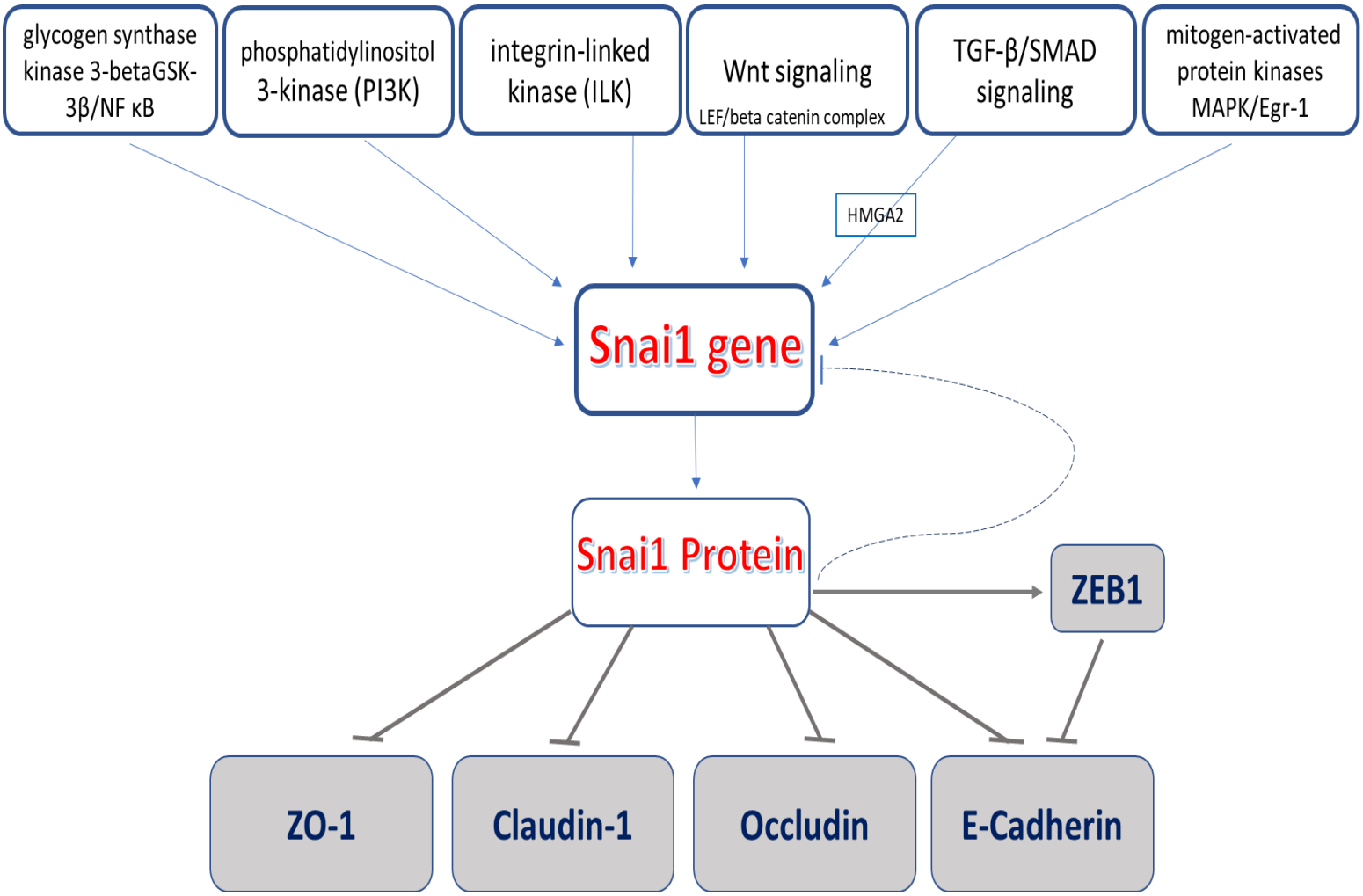

Fig. 1. Regulation of Snai1 gene expression and Snai1 transcriptional repression.
This figure represents the pathways involved in inducing Snai1 gene expression, and translation to produce Snai1 protein. The involvement of Snai1 protein in repressing E-Cadherin genes directly or indirectly through inducing another transcriptional repressor, Zeb1, that is capable of down-regulating E-Cadherin, as well as other tight junction components including Occludin, ZO-1 and Claudin-1, is shown. The dotted line represents the negative feed-back loop in which Snai1 protein acts on its gene promoter to repress its expression.

3.3.3. Snail Role in EMT

3.3.3.1. Snai1 Role in Development

The Snai1 transcriptional repressor plays an essential role in EMT induction, particularly through gastrulation in the developing mouse embryo [76]. Snai1 protein acts to maintain proper germ layer boundaries by repressing the expression within the mesoderm of regulatory genes that are involved in ectodermal development [77]. Early studies have shown that mouse embryos homozygous for a Snai1-null mutation display defects in mesoderm formation, dying shortly after E7.5 [78]. On the other hand, the epiblast-specific deletion of Snai1 at later stages of mouse embryonic development results in normal gastrulation, but results in a defect in left–right asymmetry determination and cardiovascular development [79, 80].

Additional studies using an endothelial cell-specific Snai1 loss-of-function conditional knockout mouse model have revealed an early embryonic lethal phenotype with marked defects in vascular remodelling morphogenesis, which is correlated with disruptions in arterial–venous specification. This indicates that Snai1 is a required regulator that controls vascular development during embryogenesis [81].

Moreover, during embryonic development in vertebrates, *Snai1* genes have been shown to induce EMT in the epithelial cells of the neuroectoderm which give rise to migratory neural crest cells [82]. Furthermore, conditional gain- and loss-of-function experiments have provided evidence that Snai1 is required for the early specification and migration of the neural crest. The induction of the *Snai1* gene in *Xenopus* embryos is sufficient to trigger the expression of all neural crest markers, including *Slug* in both embryos

and in animal caps. This indicates that *Snai1* is responsible along with *Slug* for neural crest specification [83, 84].

Together, these findings show that the *Snai1* gene is expressed differentially through various steps in the developing embryo, indicating the crucial role of Snai1 inducing down-stream regulatory genes during embryogenesis.

3.3.3.2. Snai1 Role in Cancer

Generally, expression of Snai1 is positively correlated with tumor grade, recurrence, metastasis and poor prognosis in various tumors. In addition, loss of E-Cadherin expression is considered to be a marker of a poor clinical outcome since E-Cadherin repressors are identified as markers of malignancy and targets for anti-invasive drugs [85].

As a critical regulator of multiple signaling pathways that lead to EMT, Snai1's expression is closely related to cancer metastasis. For example, some studies investigated the integral role of Snai1 for lymph node metastasis in human breast carcinoma showed that specific silencing of Snai1 would decrease mesenchymal markers and other metastasis features of breast cancer [86]. Other groups demonstrated that genes that are involved in lung metastasis are direct or indirect targets of Snai1 such as ID1, SPARC or MMP2 [87]. Furthermore, metastatic lesions of ovarian cancer show elevated levels of Snai1 expression [88].

Interestingly, Snai1-induced EMT was found to accelerate metastasis through induction of immune-suppression. Therefore, knocking down of *Snai1* genes suppresses tumor metastasis and increases tumor-infiltrating lymphocytes and systemic immune response in cancer patients. This indicates that Snai1 could be a potential target for preventing metastasis [89].

The role of Snai1 in cancer goes beyond metastasis to its role as a potent survival factor. Snai1-expressing cells are resistant to programmed cell death elicited by DNA damage, thus promoting tumorigenesis [74].

3.3.3.3. Snai1 Role in Fibrosis

Researchers have investigated the role of Snai1 in EMT inducing fibrotic progression in liver. *In vivo* experiments using a mouse model of acute liver fibrosis show that *Snai1*gene triggers the genes programs that are responsible in multiple aspects of fibrogenesis. Snai1 expression is highly upregulated during the process of tissue remodeling, Snai1 controls the steps that characterize the progression of fibrotic liver disorder [90].

In kidneys, Snai1 induces both developmental and pathological EMT. In some studies, investigating the role of Snai1 on kidney fibrosis, adult transgenic mice that express tamoxifen-inducible Snai1-ER model were used. Upon Tamoxifen administration, Snai1 activation is triggered, thus inducing EMT that leads to kidney fibrosis. Also, Snai1 is observed to be activated in patients with renal fibrosis [91].

Cardiac fibrosis is another disease in which *Snai1* genes play a role in triggering the fibrotic pathway. Snai1 expression is up-regulated in MI heart tissue relative to the non-MI heart [92]. Further studies have investigated the role of Snai1 in regulation of atrial fibrillation (AF) - induced myocardial fibrosis, this is by over expressing MiR-30a in cardiac fibroblasts that inhibits Snai1 expression, which leads to significantly increased degree of myocardial fibrosis, thus suggesting that Snai1 protein may be positively related to AF-induced myocardial fibrosis. [93]

Additionally, Snai1 expression is found to be significantly increased in ischemic reperfusion (I/R) injury of mice hearts [94]. Also, selective inhibition of Snai1 through injection of peroxisome proliferator-activated receptor-γ (PPARγ) agonist remarkably supresses collagen deposition and cardiac fibrosis in mouse I/R injury, which leads to an improvement in cardiac function and reduction of Snail and CTGF expression *in vivo* [94].

4. EMT Markers

EMT is clinically identified through various EMT markers including molecular markers and phenotypic markers. The molecular markers are the hallmarks that characterize EMT including an increased expression of vimentin and N-Cadherin, nuclear localization of β-catenin, and an elevated level of certain transcription factors such as Snail1 (Snai1), Snail2 (Slug), Twist, EF1/ Zeb1, Zeb2, and/or E47 that inhibit E-Cadherin production. EMT phenotypic markers induce an increased capacity for migration and three-dimensional invasion, as well as resistance to apoptosis [95]. Another common classification that describes the EMT markers is the distinction between the down-regulated epithelial markers and up-regulated mesenchymal markers.

4.1. E-Cadherin Characterization and Regulation

The hallmark of EMT is loss of E-Cadherin expression, its expression is regularly attenuated during EMT in embryonic development, fibrosis and tumorigeneses [96]. Functionally, E-Cadherin behaves as a tumor suppressor gene as it regulates cell polarity by mediating the binding between adjacent cells and creating intercellular complex forming epithelial sheets. Structurally, it consists of an extracellular portion that binds to an adjacent cell creating bridge-like cytoskeleton and an intracellular domain that interacts with β-catenin, which itself is linked to actin filaments via the α-catenin linker protein [97, 98].

In EMT, E-Cadherin is down-regulated either by a direct binding of transcription factors to the E-boxes on its gene promoter such as Snai1,Slug ,Zeb1 and Zeb2 or indirectly though certain repressors including bHLH proteins (Twist1 and Twist2), homeobox proteins (GSC and Six1) and others[5] .

Zeb1 and Snai1 both suppress the transcription of E-Cadherin by binding to the E-boxes in the E-Cadherin promoter. Alternatively, Snai1 can also cooperate with histone methyltransferases (HMTs) and DNA methyltransferases (DNMTs) to modulate the expression of E-Cadherin[99]. Snai1 directly interacts with the E-Cadherin promoter and recruits HDAC1, HDAC2 and the co-repressor mSin3A to silence its expression by deacetylation of histones H3 and H4 [65]. In conclusion, the phenotypic and cellular plasticity of EMT indicates that it is subject to epigenetic regulation, Together, this indicates that Snai1 silencing of *E-Cadherin* gene expression is mediated by multiple generic modifications to induce EMT [99]

II. Scleraxis

1.1. Basic Helix-Loop-Helix Proteins

Scleraxis belongs to the basic helix-loop-helix (bHLH) family of transcription factors. The bHLH proteins form a very large family of transcriptional regulators that exist in almost all eukaryotes [100] . They are involved in multiple regulatory cellular processes including cell development, differentiation and proliferation [101, 102].

Members of the bHLH superfamily contain a region of approximately 30 amino acid residues that is collectively made up from two highly conserved domains. The basic domain at the amino-terminal end of the bHLH motif binds the transcription factor protein to DNA at a consensus hexanucleotide sequence known as the E-box (CANNTG). The HLH domain is

located at the carboxy-terminal end of the bHLH motif and facilitates homo- and hetero-dimerization with other proteins [103].

The first bHLH motif-containing proteins identified were the transcription factors E12 and E47. Initially, the superfamily of bHLH proteins was classified into six classes based on their tissue distribution, dimerization potential and DNA-binding specificity [104]. Later, another classification system of four groups (A-D) was introduced which considers E-box binding sites, conservation of residues in some parts of the motif, and the presence or absence of certain domains. This system was extended to involve another two groups (E and F) as a result of genome sequencing and phylogenetic mapping [105].

Among these proteins, Scleraxis was identified as being classified in the class B bHLH category, which exhibits a wide expression pattern and preferably binds CAGCTG and CACCTG sequences. Generally, Scleraxis forms heterodimers with class A bHLH proteins, to which E12 and E47 belong, thus augmenting the ability of Scleraxis to bind to E-boxes [106].

1.2. Scleraxis Gene Expression in Different Tissues

Scleraxis protein is highly conserved amongst different species (e.g. humans, mice, rats). Scleraxis orthologs have been identified in a variety of organisms including chicken, frog, cow, horse and zebrafish. Scleraxis expression varies among tissues in multiple species, suggesting a pattern of functional expression that makes it involved in many physiological processes including development [107]. During murine embryonic development, Scleraxis is significantly expressed at the time of gastrulation almost up to embryonic day (E) 6.0 [108]. Its expression significantly increases in pre-skeletal mesenchymal cells prior to chondrogenesis, but declines during ossification [106].

Scleraxis is abundantly expressed in both stem cells and differentiated cells of tendons, and continues in later stages of tendon formation [109, 110]. Knockout of the *Scleraxis* gene in mice causes severe structural impairments in tendon-bone attachment, which is translated into impairment in the functional musculoskeletal force transmitted through limbs, tail and trunk [111]. Moreover, high levels of Scleraxis expression are also identified during bronchial cartilage and ligament formation [106]. Also, recent studies highlighted its potential role throughout periodontal formation as it showed that Scleraxis is highly expressed in human periodontal ligament (PDL) cells while it is expressed at lower levels in gingival fibroblasts. Notably, Scleraxis is used as a PDL cell marker or for cell lines that are committed to PDL fate [112, 113].

In addition, Scleraxis expression was shown to be widely distributed through the myocardium with high levels being noted throughout the pericardium [106]. Also, Scleraxis is highly expressed during valvulogenesis, in developing *chordae tendinae*; which are the cords that connect papillary muscles to the valves in the embryonic chick heart as well as in semilunar valve precursors [114].

Scleraxis expression is not restricted only to neonatal tissues; its expression extends to adult tissues: an RT-PCR analysis revealed the expression of Scleraxis in brain, heart, kidney, lung, muscle ,spleen and testis, but not in liver, ovary or prostate [115]. Also, data from our laboratory showed that in the adult rat, Scleraxis is expressed throughout the myocardium including in cardiomyocytes, cardiac fibroblasts (CFs) and myofibroblasts(MFBs) [116]. This suggests that Scleraxis could have a critical physiological and/or pathophysiological role that varies according to tissue type.

1.3. Scleraxis Role in Fibrosis

1.3.1 TGF-β1 Signaling Pathway and Fibrosis

Upon cardiac injury or myocardial infarction (MI), tissue remodeling and scar formation commence, and the level of proteins that regulate fibrotic scar development increase early during this process. Different cytokines like transforming growth factor-β1 (TGF-β1) are produced by a variety of cells. TGF-β1 serves as a regulator of inflammation, extracellular matrix (ECM) deposition as well as cell growth and differentiation. Initially, TGF-β1 deactivates macrophages to switch the infarcted heart from the inflammatory phase to scar formation phase through the Smad3-dependent pathway [117]. Also, it has been shown that TGF-β1 modulates fibroblast phenotype and gene expression, upregulates collagen and fibronectin and induces protease inhibitors to reduce matrix degradation [117 , 118]. In this pathway , TGFβ1 activates type II receptors (TβRII) at the cell surface which then activate a sequential intracellular cascade which includes Smad proteins, particularly Smad3, which are then translocated to the nucleus where transactivation of fibrotic genes occurs [119, 120].

1.3.2 TGF-β1 Induces *Scleraxis* Gene Expression

Our group has shown that treating cardiac fibroblasts with TGF-β1 increases Scleraxis gene expression [120]. In another study, we investigated the role of Scleraxis in inducing cardiac fibrosis. Following MI, we showed that collagen 1α2 gene expression is upregulated by Scleraxis in cardiac fibroblasts by trans-activating the human collagen 1α2 *(COL1A2)* gene and interacting with E-box sites on its proximal promoter. Furthermore, Scleraxis and Smad3 synergistically regulate collagen 1α2 expression, indicating the essential role of Scleraxis in TGF-β1/Smad-induced fibrotic pathway. [121, 116]. (Fig. 2)

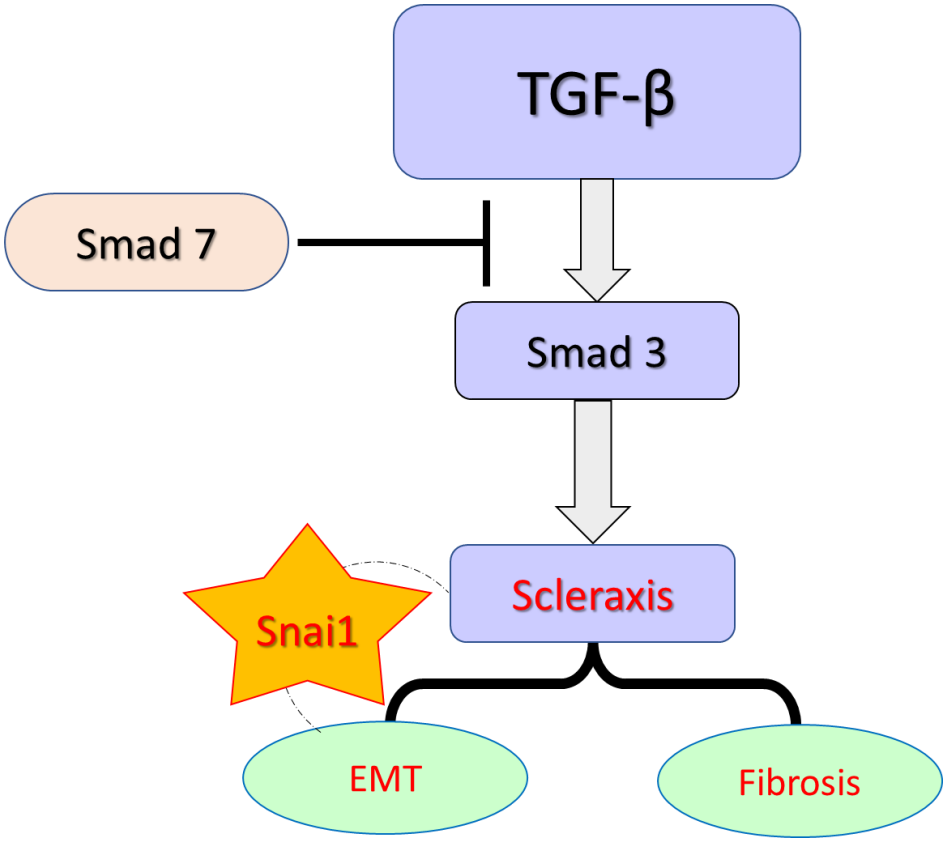

Fig. 2. Regulation of Scleraxis gene expression through TGF-β/Smad signalling pathway.
A schematic figure represents TGF-β/Smad pathway that is involved in inducing Scleraxis gene expression. Scleraxis transcription factor signaling contributes to triggering fibrosis and enhances EMT through regulating the expression of Snai1 gene; a key player in EMT. TGF-β/Smad pathway is inhibited by Smad7 gene.

Some studies showed that treating mouse muscle with TGF-β1 increases protein levels of Scleraxis and procollagen1α2. Also, treatment with TGF-β1 shifts developing limb mesoderm from chondrogenesis to fibrogenesis, which is accompanied by a Smad-mediated increase in Scleraxis [122]. Additionally, TGF-β1 treatment was found to induce Scleraxis expression in osteoblastic osteosarcoma cells and tendon fibroblasts [123, 124].

1.4. Scleraxis As a Regulator for Tissue Remodelling

A strong body of evidence indicates a role of Scleraxis in the wound healing response. Scleraxis critically controls cardiac fibroblast/myofibroblast phenotype by the direct transcriptional regulation of genes that effectively define these cells, including extracellular matrix components and α-smooth muscle actin [125]. Scleraxis is expressed by cardiac fibroblasts, the primary source of ECM production in the heart and the levels of Scleraxis protein are 4-fold higher in the infarct scar at 4 weeks post-MI scar as compared to non-infarcted cardiac tissue along with a significant increase in collagen type I and III [116, 121].

As previously mentioned, Scleraxis was identified as a novel and potent regulator for extracellular matrix genes that induce fibrotic progression by binding to the E-box consensus CANNTG on collagen 1α1 and 1α2 gene promoters in primary cardiac fibroblasts and myofibroblasts [120]. Deletion of either the basic DNA-binding domain or the helix-loop-helix protein-protein interaction domain results in a significant decrease in the ability of Scleraxis to transactivate the COL1A2 promoter [116].

Another important component of the extracellular matrix that Scleraxis induces is fibronectin. Previous studies have identified the pro-fibrotic factor TGF-β1 as an inducer of fibronectin expression, and fibronectin up-regulation usually occurs in tissue fibrosis. Our group

has showed that over-expression or knockdown of Scleraxis resulted in an increase or a decrease in fibronectin expression, respectively. Furthermore, Scleraxis was required for TGF-β1-induced fibronectin expression. We determined the mechanism by which Scleraxis acts to induce fibronectin expression: Scleraxis binds directly to the two E-boxes present in the proximal human fibronectin promoter, with a higher affinity to the second E-box, which is both sufficient and necessary for Scleraxis mediating fibronectin expression to occur [126].

Not only were the collagen 1α2 and fibronectin genes were found to be directly transactivated by Scleraxis, but also Scleraxis induces the expression of various ECM genes, for example other fibrillar collagens, proteoglycans, matrix metalloproteinases (MMPs) and numerous markers of the FB and MFB phenotype in primary cardiac proto-myofibroblasts. Moreover, Scleraxis directly transactivates the gene promoters of vimentin, MMP2 and α-SMA. [125]

Collectively,these findings demonstrate that Scleraxis is critical for directly regulating the genes responsible for cardiac fibroblast/myofibroblast phenotype conversion [125].

1.5. Scleraxis Role in Epithelial-Mesenchymal Transition

While Scleraxis promoted fibroblast to myofibroblast conversion, loss of Scleraxis attenuated myofibroblast function and gene expression. Our previous studies showed that Scleraxis null hearts exhibited a ~50 % reduction in cardiac fibroblasts number and a deficiency in matrix deposition, and also reported that epithelial markers were elevated while mesenchymal markers were reduced. This effect could be due to a failure of epithelial precursors to undergo mesenchymal transition during development – a key step in cardiac fibroblast formation. Furthermore, overexpression of Scleraxis in A549 epithelial cells induced up-regulation of

mesenchymal markers including *Snai1* and *Twist1*, and fibroblast markers including vimentin, ED-A fibronectin and type I collagen, while at the same time downregulating epithelial markers. Together, this indicates that Scleraxis could be sufficient to transactivate EMT genes and induce mesenchymal/fibroblast phenotype conversion of A549 epithelial cells. Conversely, loss of Scleraxis attenuated TGFβ-induced EMT marker expression [125].

Fate mapping studies have investigated the potential angiogenic role of Scleraxis. As the mature proepicardial organ (PEO) migrates to encapsulate the heart, it forms a uniform epithelium and a proportion of these epicardial cells undergo an epithelial-to-mesenchymal transition (EMT) and populate in the subepicardial space [127].Later, endothelial cells from coronary epithelium which are derived from the subepicarduim, populate and migrate to form ventricular capillary plexus [128, 129].

Interestingly, these populations of epicardial cells have been defined using Scleraxis along with Semaphorin as Cre-drivers in lineage tracing experiments. Together, it has been shown that Scleraxis expressing along with Semaphorin expressing progenitors in the developing proepicardial organ gives rise to the coronary vascular endothelial cells by contributing to fibroblasts and coronary endothelium as well as the vascular smooth muscle layer [130]. This suggests that Scleraxis could be a potent regulator for EMT in angiogenesis.

Additionally, early Scleraxis expression in the otic mesenchyme indicates a role as regulator in fibrocyte differentiation and gross cochlear morphogenesis. Since mesenchymal cells originate from the paraxial mesoderm, they begin to condense around the otocyst at E10 and the closest mesenchymal cells to the developing otic epithelium differentiate to become otic fibroblasts, whilst further cells aggregate and differentiate to form the cartilaginous cells of the otic capsule.

This series of complex interactions between the ectodermal mesenchyme and epithelia of the cochlear duct develop what is called the inner ear [129, 131].During these interactions ,a consistent robust expression of Scleraxis along with TGF-β1 is present, indicating their correlation with the pathway that regulates cochlear EMT. It's worth mentioning that mesenchymal-epithelial interactions throughout cochlear development are also crucial for otic fibrocyte differentiation and gross cochlear morphogenesis [132].

During embryogenesis, heart valves and leaflets originate from mesenchymal precursor cells within the endocardial cushions that is formed as a result of endothelial-to-mesenchymal transition (EndMT) in the atrioventricular canal [1, 114] .Scleraxis is highly expressed during the beginning of valve remodeling when it is functionally required for the development of tissues of high mechanical demand [3].This indicates the role of Scleraxis in EMT during valvulogenesis.

❖ Rationale

Published data from our group suggest that Scleraxis overexpression is sufficient to induce EMT. In brief, Scleraxis overexpression in A549 epithelial cells induces up-regulation of mesenchymal markers including Snail1 and Twist1, and fibroblast markers including vimentin, ED-A fibronectin and type I collagen, while at the same time downregulating epithelial markers [125].

Moreover, Scleraxis knockout mice show a significant reduction of Snai1 mRNA abundance and evidence of overall cardiac pathological hypotrophy, thus abnormal reduction of cardiac size due to the reduced number of fibroblasts, matrix deficiency and reduced volume of cardiac myocytes. RT-PCR analysis further, showed that mesenchymal marker (Snai1, Twist1, Zeb1) levels were significantly down-regulated, while epithelial markers (Dsp, Par3, Claudin1) were increased [125]. Therefore, loss of fibroblasts could be potentially due to an alteration in the cell fate of fibroblast precursors. This builds on the hypothesis that fibroblasts originate from epithelial cells through EMT. These epithelial progenitors originate from the epicardial cells surrounding the heart. In other words, EMT generates new mesenchymal cells that expand the pool of fibroblasts available for phenotype conversion [133]. The conversion of fibroblasts to myofibroblasts has also been characterized as a continuation of the developmental EMT process that gives rise to the majority of the fibroblasts in the myocardium.

Further studies show that overexpression of Scleraxis in NIH 3T3 cells resulted in a dose-dependent increase in the luciferase Snai1 promoter reporter activity accordingly [125]. Additionally, overexpression and knockdown experiments in cardiac proto-myofibroblasts revealed the potential regulation of Scleraxis of mesenchymal marker genes, especially, *Snai1* [125]. However, the exact mechanism of this regulation is still not clear.

Snai1 transcription factor is the main inducer for EMT and fibroblast pheno-conversion in development, fibrosis and cancer progression, by downregulating the adhesion molecule E-Cadherin and supressing its action.

Taking all of this in consideration, we endeavoured to reveal whether Scleraxis interacts with the Snai1 gene promoter to regulate its transactivation, and how this contributes to triggering molecular signaling and responses that characterise EMT and fibroblast determination.

❖ **Main Hypothesis**

Scleraxis regulates Snai1 gene expression by directly binding to the E-boxes present within the Snai1 gene promoter, thus increasing Snai1 expression that in turn represses E-Cadherin expression and induces the pathways associated with EMT.

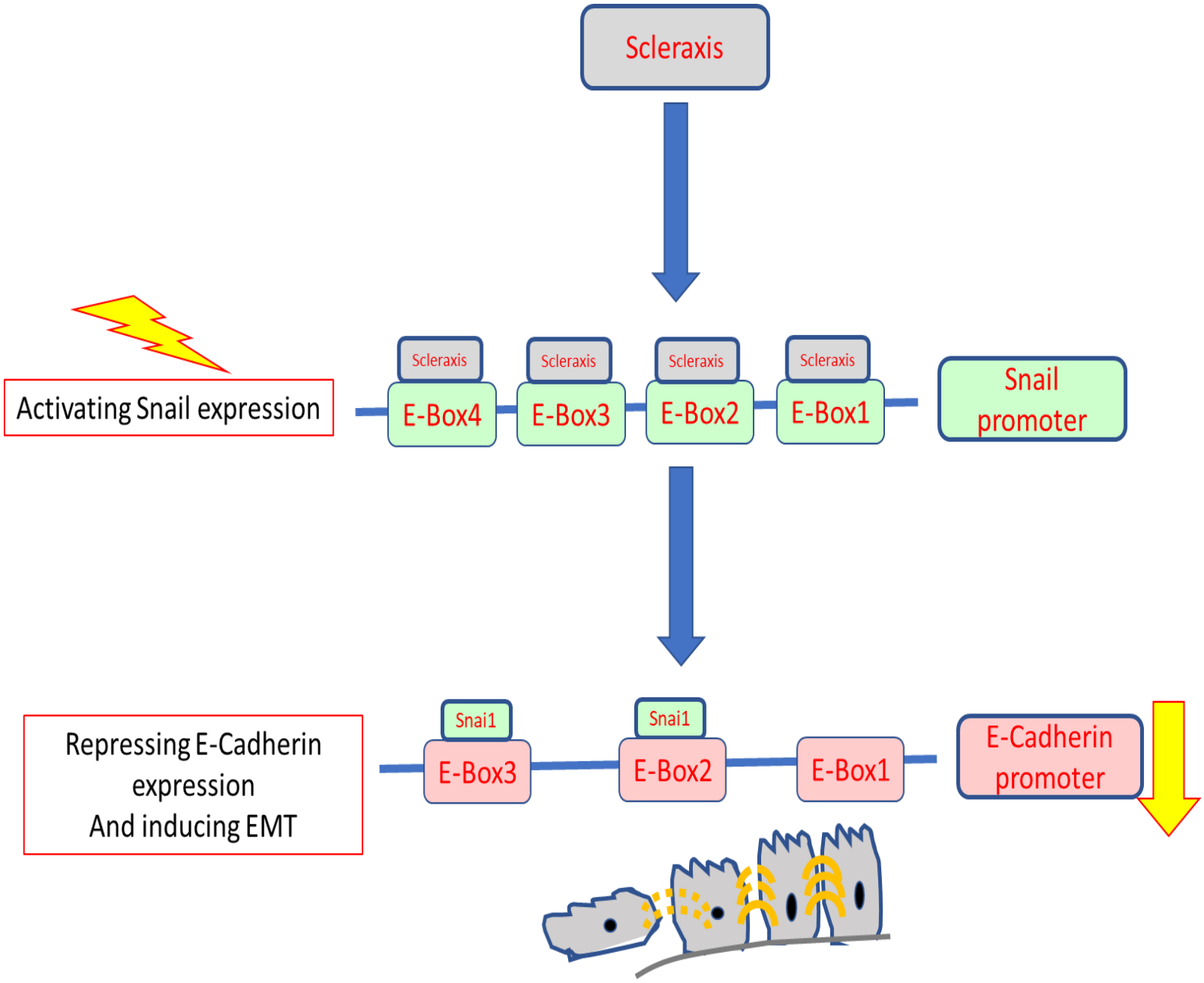

Fig. 3. Overall Visual Hypothesis

Scleraxis expression upregulates Snai1 expression directly through binding to one or more E-boxes present within the Snai1 gene promoter. Furthermore, this interaction triggers production of Snai1 protein that interacts to repressor E-boxes present within the E-Cadherin gene promotor to induce cell responses that have been associated with EMT (eg. Loss of cell-cell adhesion)

❖ Objectives

1. *Assay Snail1 protein abundance in relation to Scleraxis overexpression or knock down in A549 epithelial cells.*

Various studies have confirmed the role of Scleraxis in regulating the expression of key target genes, promoting a tissue-specific function. For example, this basic helix-loop-helix transcription factor directly transactivates ECM genes in cardiac fibroblasts, playing a potential role in cardiac fibrosis [125], while Scleraxis also regulates tendon formation during embryogenesis [134], suggesting that the role of Scleraxis is to determine the fate of matrix-producing cell types, which vary according to tissue type. To support what was already published by our group that it induces, i.e. Scleraxis-mediated expression of Snai1 in mouse cardiac proto-myofibroblasts at mRNA level [125]. We will test whether Scleraxis regulates Snai1 protein abundance. We will also investigate whether this regulation of Snai1 expression by Scleraxis is similar in A549 cells to other cell types.

2. *Identify the molecular mechanism by which Scleraxis regulates Snai1 gene expression.*

Scleraxis, as part of the bHLH superfamily, binds to a consensus hexanucleotide sequence known as the E-Box (CANNTG) on the target gene promoter and transactivates its expression. Therefore, we aim to define the specific binding sites for the Scleraxis protein in the Snai1 gene promoter.

The E-boxes present on the Snai1 gene promoter could be potential interaction sites for Scleraxis protein as well as other bHLH superfamily proteins. In addition, E-boxes could have different functions per the type of factor binding to each E-box. For example, Snai1 undergoes self-inhibition by binding to the E-box present in its promoter (at −146 with respect to the transcription start) inhibit its activity [73].

3. *Investigate if TGF-β1 mediates Snai1 gene expression via Scleraxis regulation.*

Previous studies have identified that TGF-β controls the expression of Snail1 in epithelial cell lines [48]. TGF-β1 is also a pro-fibrotic factor that induces the expression of Scleraxis in cardiac fibroblasts as well as during embryogenesis [121]. Therefore, we will assess the potentially essential role for Scleraxis in TGF-β mediated induction of Snai1 expression by using a protein assay.

4. *Correlate the effect of Scleraxis regulation of Snai1 gene expression with induction of markers for epithelial mesenchymal transition.*

As previously mentioned, Snai1 plays a significant role in inducing EMT through downregulation of the adhesion molecule E-Cadherin that alters the stability of epithelial cells [75]. Therefore, we will evaluate the EMT markers through Scleraxis upregulation while knocking down Snai1 to assess the role of Scleraxis in EMT induction.

❖ Materials & Methods

A. NIH 3T3 cell culture and luciferase assay:

NIH 3T3 fibroblasts (CRL-1658, ATCC, USA) were plated in 6-well cell culture plates and maintained in growth medium (Hyclone DMEM High-Glucose (SH30022.01, Thermo Fisher Scientific, Canada) supplemented with 10% FBS and 1% penicillin/streptomycin antibiotic) for 24 hr. Cells were then maintained in Opti-MEM (Gibco Life Technologies) while being transfected at 65% confluence using Lipofectamine 3000 transfection reagent (Invitrogen,USA) as per manufacturer`s instructions. Each sample was co-transfected with a total of 1000 ng of DNA, as such with 500 ng of an intact Snai1 promoter (Snail-PGL4) or 500 ng of one of the mutated ΔE1-ΔE4 Snai1 promoter luciferase reporter constructs with the addition of either Scleraxis expression vector (Scleraxis-pECE) or an empty vector (pECE) as a basic control, plus 5ng Renilla luciferase expression vector (pRL) which was used as a transfection control. Cells were washed with PBSx1 then harvested and lysed 24 hours after transfection, and then luciferase assay was performed. The luciferase activity of each sample was assayed using the Dual Luciferase Reporter Assay System (Promega, USA) on a Glomax Multi+ Detection System (Promega, USA). Mutations of the E boxes (ΔE1-ΔE4) within Snai1 promoter luciferase reporter were generated using a Quik Change II Site Directed Mutagenesis kit (Stratagene) and nested PCR using specific primers, and was confirmed through sequencing. (same as cold primers in Table 2)

B. A549 cell culture and treatment (Scleraxis overexpression and rescue):

Adenocarcinoma human epithelial A549 cells (ATCC® CCL-185™) were counted via hemocytometer and cells were plated in 6 well cell culture plates, approximately 120000 per well. 24 hr after plating, cells were serum starved (DMEM only) for 24 hr prior to infection.

Cells were then infected with overexpression adenovirus encoding green fluorescent protein (AdGFP) control or Scleraxis (AdScx) at 100 multiplicity of infection (MOI) for 48 hr until being harvested, or with knockdown adenovirus encoding for LacZ-targeting shRNA (AdshLacZ) or Scleraxis-targeting shRNA (AdshScx) for 48 hr at 200 MOI, then treated with 10 ng/ml TGF-β1 or vehicle for additional 24 hrs. Cells were then washed with PBSx1, lysed and harvested for protein.

C. Cell treatment for EMT experiments (using Snail siRNA)

Pre-designed silencer siRNA's along with lipofectamine RNAiMAX were obtained from Thermofisher (Table 1). A549 cells were plated in 6-well plates at ~70% confluency and transfected with either Snai1 siRNA or scrambled siRNA (50 pmol/well) in serum-free medium according to manufacturer's instructions for 24 hours. Cells were then infected with either AdScx or AdGFP at MOI 100 for an additional 48 hours before harvesting cells for protein determination.

Table1: siRNA details

CAT #	Product Name
4390843	SIL. SEL. NEG. CONTROL #1 5 NM EACH (Scrambled SiRNA)
AM16708 (4392420)	SILENCER PRE-DESIGNED SIRNA 5NMOL ID Assay s13185 Chr. 20: 49982976 - 49988886 on Build GRCh38 SIRNA Location 1211 targeted exons 3
13778030	Lipofectamine RNAIMAX

D. Wound Heal Assay (Scratch Assay)

A549 cells were plated in 6-well tissue culture dishes at 100000 cells/well and allowed to attach overnight. Following 24 hr of serum starvation, cells were infected with either AdGFP or AdScx adenovirus at 100 MOI for 48 hr. A line was made on the underside of each well and a scratch introduced over this line using a medium pipette tip. Cells were washed 3 times with 1X PBS to remove excess cells from wounding, and treated with starvation media. Pictures of initial wounds (0 hours), as well as after 24 hr were taken using a Cell Citation Imager, phase contrast at 20X objective.

E. Western Blots

A549 cells in 6-well plate were infected with either AdScx or AdGFP at MOI 100. 48 hrs after treatment, cells were harvested and lysed using RIPA buffer 150µl/per well (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Na-deoxycholate, 1% Triton-X 100, 0.1% SDS), PMSF 10 µl, DTT 2 µl , Mini Protease Inhibitor Cocktail (Roche, Canada)10 µl. Protein concentration was determined via Bradford assay (measured at 600 nm wavelength) using Coomassie Blue Protein Assay Reagent (1856209, Thermo Fisher Scientific, Canada) and bovine serum albumin standards (Thermo Scientific, 23209). Samples were prepared using 2X Laemmli buffer, boiled for 5 min at 100°C, and 25 µg protein was loaded into 12% polyacrylamide gels in SDS-PAGE buffer then transferred to PVDF membranes (Pall, USA). The membrane was probed using primary antibodies against: Scleraxis (Rabbit monoclonal, custom-made), E-Cadherin (Abcam –Rabbit monoclonal), Snai1 (Rabbit monoclonal- Cell signaling), and α-Tubulin (Mouse monoclonal -DSHB) as a loading control. Then blots were incubated with appropriate HRP-conjugated mouse or rabbit secondary antibodies. Antibodies

were detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, USA) and blue X-ray film (Thermo Scientific, Canada).

F. Electrophoretic mobility shift assay (EMSA)

HEK293 cells were plated in 10 cm^2 cell culture plates and transfected with 3 μg Scleraxis expression vector. After 48 h, nuclear and cytoplasmic proteins were isolated using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, USA). 2 μl nuclear extracts were used in binding reactions as required. Biotin-labeled oligonucleotides and cold probes were synthesized commercially (Integrated DNA Technologies) (Table 2). Assays were performed using a Lightshift Chemiluminescent EMSA kit (Pierce Biotechnology, USA) as per manufacturer's instructions. Binding reactions were incubated at room temperature for 30 min in buffer containing 10 mM Tris pH 7.5, 100 mM KCl, 0.5 mM EDTA, 5% glycerol, 50 ng poly (dI-dC), 5 μg bovine serum albumin (BSA) and 20 fmol biotin end-labeled probe. For competition experiments, a 250-fold molar excess (5 pmol) of unlabeled probe was included in the binding reactions and incubated for 20 min before addition of the labeled probes. For supershift reactions, either 3 μg anti-Scleraxis antibody or IgG as a control was incubated overnight at 4 °C before the addition of the biotin-labeled probes to the reaction. DNA-protein complexes were run on a non-denaturing 6% native polyacrylamide gel at 100 V for 1 h, and then transferred to positive charged nylon membrane. Chemiluminescent complexes were detected using CL-XPosure clear blue film (Thermo Scientific, Canada).

Table 2: Snail EMSA + WT/Mutated Primers_Cold/Biotin labelled (human)

E-Box 1 – (FOR)	AGGCCACTCCCCGAGCAGGTGCGCCGGCTGCTGGCC
(REV)	GGCCAGCAGCCGGCGCACCTGCTCGGGGAGTGGCCT
Mutant E-Box 1 –(FOR)	AGG CCA CTC CCC GAG CTC AGG CGC CGG CTG CTG GCC
(REV)	GGC CAG CAG CCG GCG CCT GAG CTC GGG GAG TGG CCT
E-Box 2 – (FOR)	GCGCCGGCCGGGACACCTGACCTTCCGACGCCCG
(REV)	CGGGCGTCGGAAGGTCAGGTGTCCCGGCCGGCGC
Mutant E-Box 2 –(FOR)	GCG CGC CGG CCG GGA CTC AGG ACC TTC CGA CGC CCG
(REV)	CGG GCG TCG GAA GGT CCT GAG TCC CGG CCG GCG CGC
E-Box 3 – (FOR)	AGGAAAGGGACACCGCACGTGGCTCTCGGCGACTTG
(REV)	CAAGTCGCCGAGAGCCCTAGGCGGTGTCCCTTTCCT
Mutant E-Box 3 –(FOR)	AGG AAA GGG ACA CCG CCT AGG GCT CTC GGC GAC TTG
(REV)	CAA GTC GCC GAG AGC CCT AGG CGG TGT CCC TTT CCT
E-Box 4 – (FOR)	TTACGGAGCGCCCAGCACGTGTGGGGCACTGTACCT
(REV)	AGGTACAGTGCCCCACCTAGGCTGGGCGCTCCGTAA
Mutant E-Box 4 –(FOR)	TTA CGG AGC GCC CAG CCT AGG TGG GGC ACT GTA CCT
(REV)	AGG TAC AGT GCC CCA CCT AGG CTG GGC GCT CCG TAA

G. Chromatin immunoprecipitation assay (ChIP)

Adult human cardiac fibroblasts (HCFa cell applications Cat 306-05a) (~ 8×10^5 cells) were grown in 10 cm^2 dishes and fixed by addition of 280 µl 37% formaldehyde to 10 ml of culture media for 10 min at 37 °C. The cells were then washed with ice-cold PBS containing protease inhibitors (1x) and PMSF (1 mM), and harvested by scraping in 200 µl SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8). Cell lysates were sonicated to shear DNA to 200–500 bp fragments. A 1% aliquot of this cell lysate was used as an input control. Samples were pre-cleared with 75 µl bovine serum albumin (0.5 mg/ml)/herring sperm DNA (200 µg/ml)/protein A-agarose mixture at 4 °C overnight with rotation to reduce non-specific background. Lysates with the sonicated chromatin collected after brief centrifugation were incubated at 4 °C overnight with 10 µg anti-Scleraxis antibody or rabbit IgG (Santa Cruz Biotechnology) as a negative control. The DNA-protein immune complexes were precipitated,

washed with low salt , high salt, LiCl and TE buffer respectively, eluted using elution buffer (1%SDS, 0.1M $NaHCO_3$) and subsequently reverse cross-linked by heating at 65^0 C for 4 hours, then DNA recovered by phenol/chloroform extraction. DNA samples were subjected to qPCR amplification using primers specific to the Scleraxis-binding E box sequences in the human Snail gene promoter. Primers encompassing a region of the human GAPDH promoter were used as a negative control. Fold enrichment was calculated (ΔCt) and represents the ratio of protein bound DNA (Scleraxis protein bound to Snai1 promoter chromatin pieces) to a negative control (non-specific IgG) normalized for the input. A region of the GAPDH promoter (human) was amplified to represent a negative control for the experiments; no difference was observed for Scleraxis binding to GAPDH promoter ($P>0.05$; $n=4$).

Table 3: qPCR Primers used in CHIP(HUMAN)

5'- GAPDH promoter (Human)	**AGAAGGCTGGGGCTCATTTG**
3'- GAPDH promoter (Human)	**AGGGGCCATCCACAGTCTTC**
5'- Scleraxis (Human)	**GTGAACACGGCCTTCACGG**
3'- Scleraxis (Human)	**CTGCGAATCGCTGTCTTTC**

❖ Results

1. Scleraxis overexpression induces Snai1 expression

Our group has demonstrated the role of Scleraxis upregulation in increasing Snai1 mRNA in both A549 cells and in primary mouse cardiac proto-myofibroblasts; fibroblasts isolated from mouse heart then passaged once to P1(passage 1) proto-myofibroblasts [125]. I confirmed and extended these findings by measuring the protein level of Snai1 in correlation to Scleraxis overexpression in human alveolar basal epithelial A549 cells, assayed by Western blot; Scleraxis over-expression induced Snail1 expression (Fig. 4).

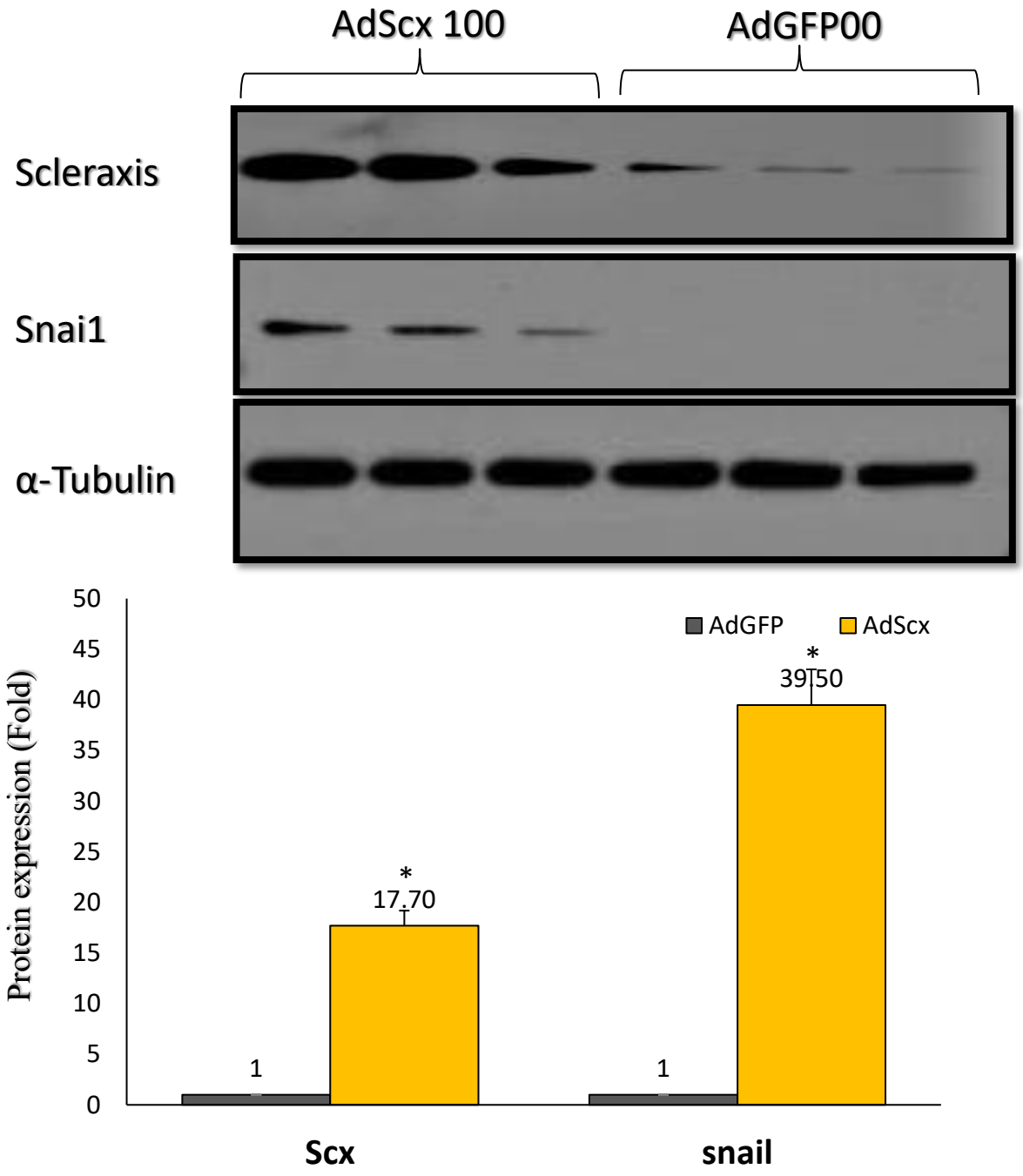

Fig 4.: Scleraxis upregulates Snai1 gene expression.

Western blot images and analysis showing detection of Scleraxis , Snai1 and α-tubulin (as a loading control) proteins lysates from A549 cells infected with either AdScx (MOI 100) or control AdGFP (MOI 100) and analysed 48 hr post infection. N=3. *P<0.05 vs AdGFP.

2. Scleraxis binds to Snai1 gene promoter *in vivo*

In order to determine whether Scleraxis binds to the Snai1 gene promoter, we performed chromatin immunoprecipitation (ChIP) assay. Using human cardiac fibroblasts, our data demonstrate that Scleraxis directly binds to the promoter region of the *Snai1* gene where E-boxes are present (Fig. 5). However, it is not clear which of the E-boxes are bound by Scleraxis, since ChIP resolution is determined by the size of the immunoprecipitated DNA fragments, typically 200 to 500 bp, and the gap between each E-box present on the Snai1 promoter is less than 500 bp.

As a result, we confirmed that Scleraxis binds to the Snai1 promoter, but we are still uncertain which of E-boxes is actually bound by Scleraxis. To answer this question, we performed luciferase reporter assays using reporters in which each Snai1 E-box was mutated.

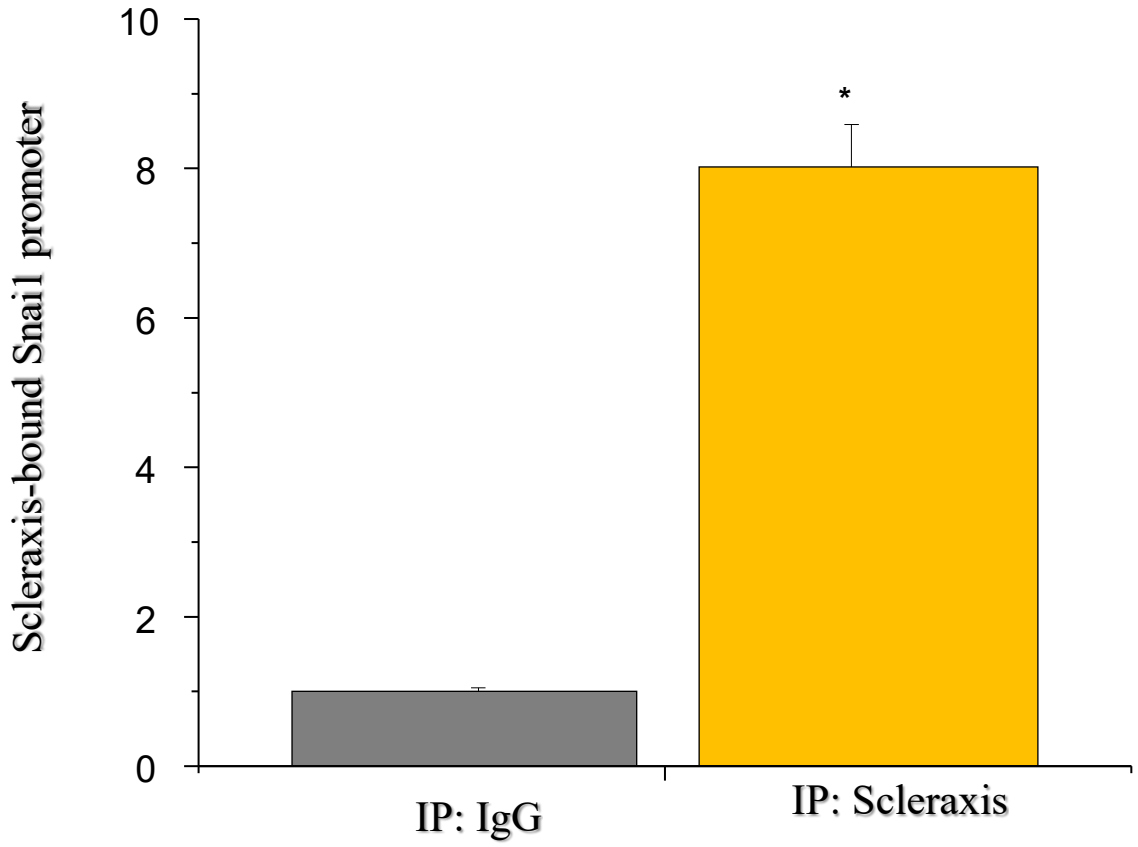

Fig 5: Scleraxis directly interacts with the E-boxes in the Snai1 gene promoter
Human cardiac myofibroblasts were used to perform chromatin immunoprecipitation using anti-Scleraxis antibody or IgG as a control. qPCR human primers were used to amplify the Snai1 promoter sequence. Results of n = 3 independent experiments, *$p \leq 0.05$ vs IP:IgG

3. Transactivation of the human Snai1 promoter by Scleraxis is mediated by multiple E-boxes

As Scleraxis is a part of the bHLH family, it regulates the transcription of different genes through binding to E-Box binding sites present within its target gene promoters. We identified the presence of four putative E-Boxes within the Snai1 gene promoter which could be potential binding sites for Scleraxis regulation.

To elucidate the mechanism by which Scleraxis transactivates the expression of Snai1, luciferase reporter assays were performed in NIH 3T3 fibroblasts. We sub-cloned the Snai1 gene promoter into pGL4.10 luciferase reporter vector and validated it by sequencing. Later, mutation analysis studies were performed in which each of the E-boxes was mutated separately and confirmed by sequencing (Fig. 6).

Our data show that overexpressed Scleraxis significantly transactivates intact Snai1 promoter compared to empty vector (Fig. 7). Moreover, the luciferase activity of each of the point mutated E-boxes (2, 3, or 4) was reduced (Fig. 7). This suggests that the mutation of these E-boxes disrupts the binding of Scleraxis to these sites, thus altering Scleraxis mediated-transcriptional activity of *Snai1* gene.

Interestingly, the luciferase activity of the mutated E-Box1 shows approximately 2-fold higher activity compared to the intact Snai1 gene promoter. Our data is consistent with the finding that Snai1 protein binds to its own promoter at this specific site E-Box1 and represses its expression as previously reported [73]. Overall, this experiment demonstrates that Scleraxis is a potent transcriptional regulator for Snai1 gene expression via interaction with the three E-boxes found within its gene promoter.

E1 : CAGGTG

ΔE1: CTCAGG

```
38    GAGGGGCGGGGCCTTATCTGCCACGCCCCTTTGTCACCTTCGCGCCAATCGGAGGCTCGT  97
      |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
991   GAGGGGCGGGGCCTTATCTGCCACGCCCCTTTGTCACCTCCGCGCCAATCGGAGGCTCGT  932

98    CTCCGCCGAAGGCCACTCCCCGAGCTCAG-GCGCCGGCTGCTGGCCGGGCTCGCGCCGCG  156
      |||||||||||||||||||||||||  | ||||||||||||||||||||||||||||||
931   CTCCGCCGAAGGCCACTCCCCGAGC-AGGTGCGCCGGCTGCTGGCCGGGCTCGCGCCGCG  873
```

E2: CACCTG

ΔE2: CTCAGG

```
260   GGGACCCCGGCAGGACGCCGCGCGCCCACCGGCTCAGCGCCCGGTGGTCTGAGCGCTTCT  319
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
751   GGGACCCCGGCAGGACGCCGCGCGCCCACCGGCTCAGCGCCCGGTGGTCTGAGCGCTTCT  692

320   GACGCCCCCTGGCGCTGCGCGCCGGCCGGGACTCAGGACCTTCCNACGCCNNCCCC-GCC  378
      |||||||||||||||||||||||||||||| |  |||||||| |||||  |||| |||
691   GACGCCCCCTGGCGCTGCGCGCCGGCCGGGACACCTGACCTTCCGACGCCCGCCCCCGCC  632
```

E3: CACGTG

ΔE3: CCTAGG

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443   GGGCGCTCCGTAAACACTGGATAAGGGAAGGAACGGGTGCTCTTGGCTAGCTGGGCCAGG  502
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
926   GGGCGCTCCGTAAACACTGGATAAGGGAAGGAACGGGTGCTCTTGGCTAGCTGGGCCAGG  985

503   CTGCTTTGCAAAAAGGCCGTGGCATTTCAAGTCGCCGAGAGCC-CTAGGCGGTGTCCCTT  561
      |||||||||||||||||||||||||||||| ||||||||||  |   ||||||||||||
986   CTGCTTTGCAAAAAGGCCGTGGCATTTCAAGCCGCCGAGAGCCACGT-GCGGTGTCCCTT  1044
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E4 : CACGTG

ΔE4 : CCTAGG

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383   GCTCTGAGTGTTCTGTCCGGGGCTGTGCCCTGGCCCCAGGTACAGTGCCCCAC-CTAGGC  441
      |||||||||||||||||||||||||||||||||||||||||||||||||||||| |   ||
866   GCTCTGAGTGTTCTGTCCGGGGCTGTGCCCTGGCCCCAGGTACAGTGCCCCACACGT-GC  924

442   TGGGCGCTCCGTAAACACTGGATAAGGGAAGGAACGGGTGCTCTTGGCTAGCTGGGCCAG  501
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
925   TGGGCGCTCCGTAAACACTGGATAAGGGAAGGAACGGGTGCTCTTGGCTAGCTGGGCCAG  984
```

Fig. 6: Sequencing of the point mutated E-boxes on the Snai1 gene promoter

Sequencing of mutated E-box sites on the Snail gene promoter ΔE1-E4 (red box) which mis-matches the sequence of the E-box that was originally present in the intact Snai1gene promoter, which are critical sites for Scleraxis binding .

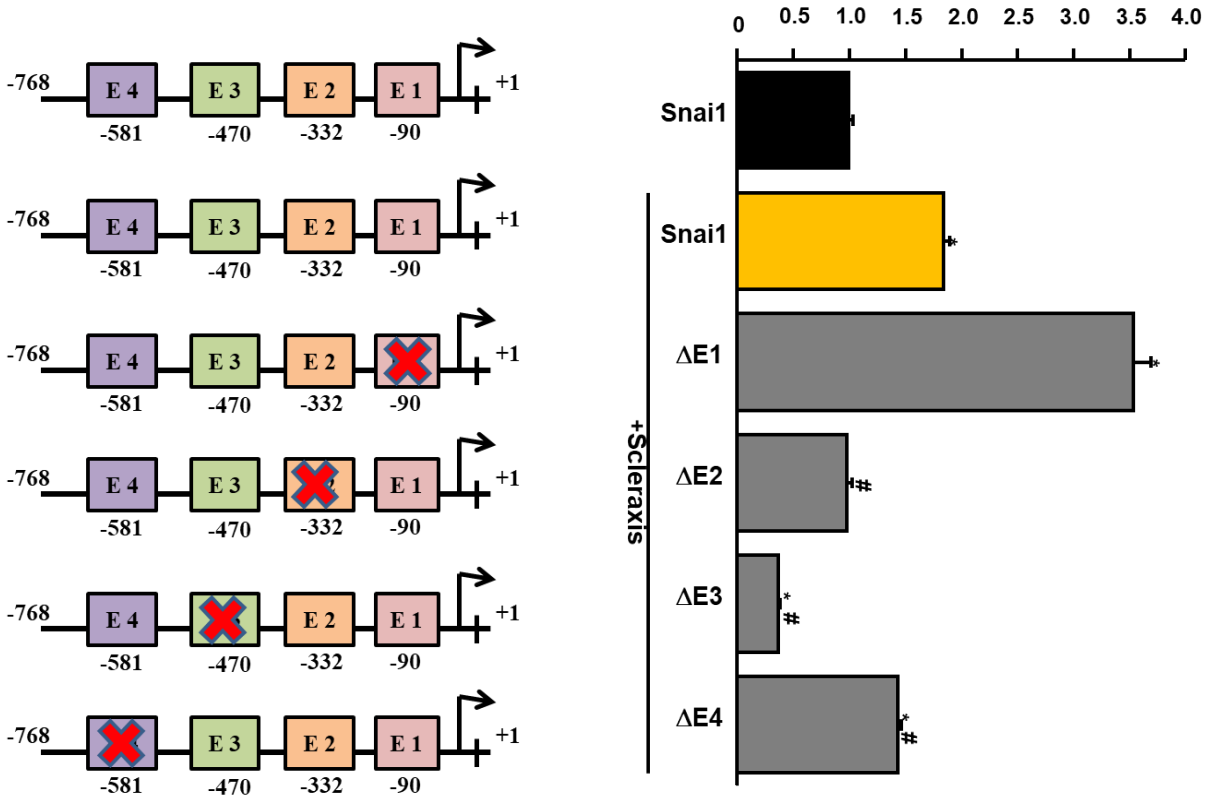

Fig. 7: Scleraxis transactivates the human Snai1 gene promoter by directly binding to the multiple E-boxes present on its promoter

A Luciferase reporter assay was performed using NIH-3T3 fibroblasts,which were transfected with WT (Snai1) or mutated (ΔE1 – ΔE4) Snai1 promoter (Snail-pGL4.1) luciferase reporter constructs in addition to either a Scleraxis expression vector (Scleraxis-pECE) or an empty vector control (pECE). Renilla luciferase (PRL) was used as a transfection control. 24 h after transfection, luciferase assays were performed. Results of n=3 independent experiments were normalized to control and Renilla; mean±SEM, *p<0.01 vs. Snai1, #p<0.01 vs. Snai1+scleraxis.

4. Scleraxis interacts with the E-boxes of the human Snai1 gene promoter

To confirm that Scleraxis directly binds to the E-boxes on Snai1 gene promotor, electrophoretic mobility shift assays (EMSA) were performed using nuclear extracts from Scleraxis over-expressing HEK293 cells and biotin-labelled probes corresponding to each E-box and its mutated biotin-labelled oligo. Different concentrations of Scleraxis polyclonal antibody were used to cause a supershift (1 μg – 3μg).

We observed that there are no shifted bands and no reduction in the cold-competition lane in both biotin-labelled probes of either intact or mutated E-box 1 (Fig. 8A). This finding confirms that E-box 1 is not bound by Scleraxis.

In contrast, we observed a shifted complex on E-boxes 2, 3 and 4, and these interactions were lost when the E-boxes were mutated (Fig. 8B-D). Moreover, a clear supershift of the complex was observed when we further incubated the protein-DNA complex with 3 μg of Scleraxis polyclonal antibody, which is represented in E-boxes 3 and 4 (Fig. 8C-D).

In combination, our data identifies the E-boxes on the Snai1 gene promoter to which Scleraxis binds to transactivate Snai1 gene expression.

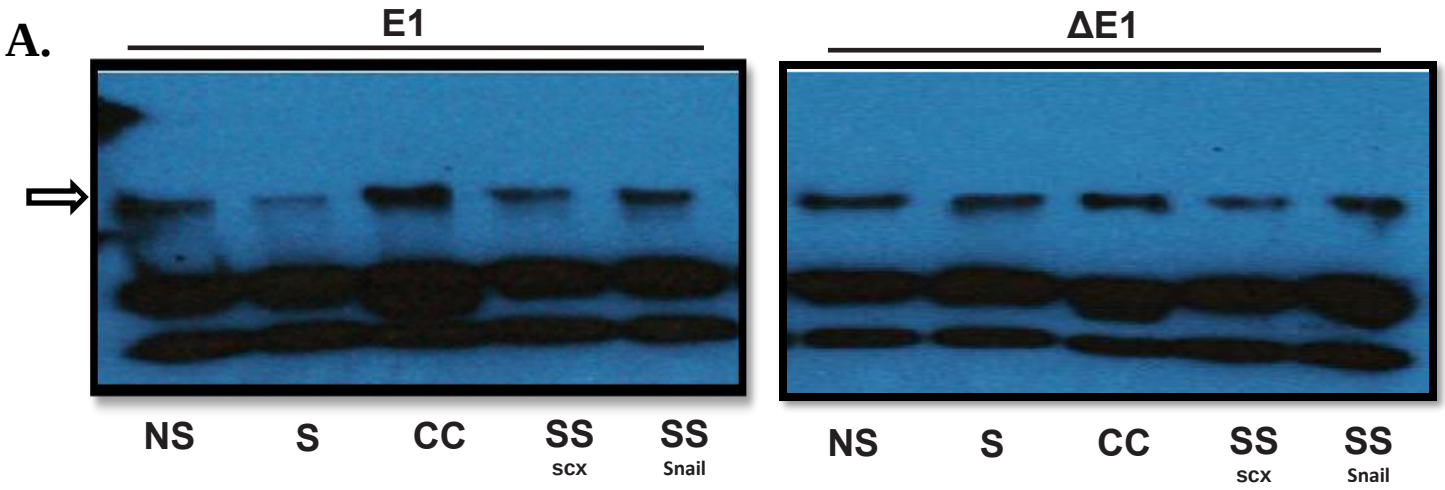

Fig. 8: Scleraxis interacts with the E-boxes of the human Snai1 gene promoter.

The ability of Scleraxis to bind to the oligonucleotides incorporating each of the four E-boxes of Snai1 gene promoter was tested by electrophoretic mobility shift assay (A-D). Biotin-labeled oligonucleotide probes corresponding to either intact E-boxes (E1-4) or mutated E-boxes (ΔE1-ΔE4) were incubated with Scleraxis-transfected (S) or non-transfected (NS) HEK293 nuclear lysates, resulting in either a shift of oligonucleotide-protein complex (black arrow) or no shift. A fainter band can be present using unlabeled oligonucleotide (CC) which used to cold compete the Scleraxis-DNA complex. Super-shifts (SS) were performed using anti-scleraxis polyclonal antibody.

(A) No interaction of Scleraxis with E-Box1; representative unspecific bands (empty arrow) were similar on both intact E1 and ΔE1. On the last lane (SS Snai1) the complex was incubated with Snai1 antibody to test the possibility of a supershift resulting from indogenous intranuclear lysates of Snai1, but result shows no interaction.

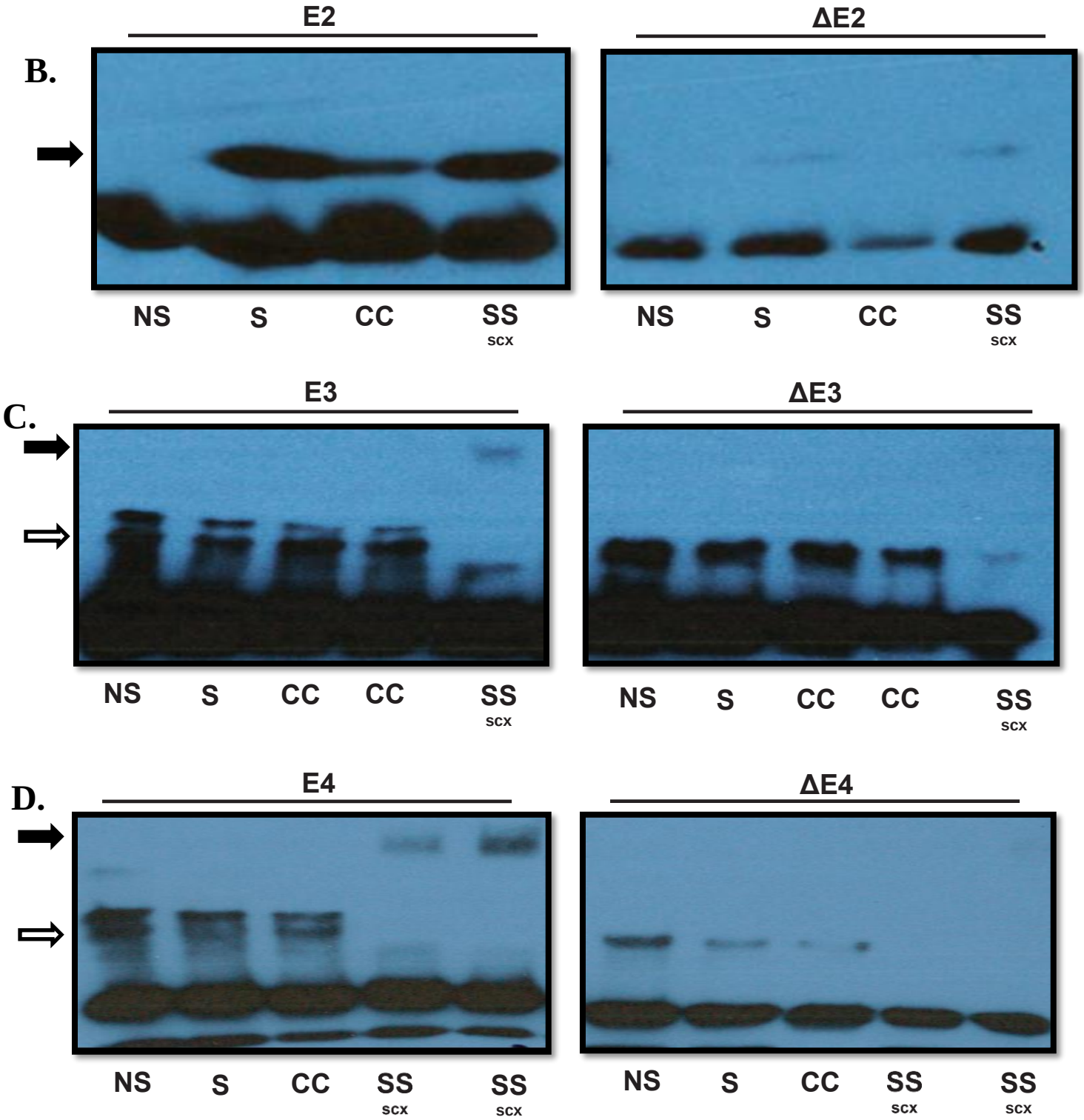

Fig. 8 :(B-D) Scleraxis directly interact with three E-boxes on the human Snai1 gene promoter.

(B-D) Scleraxis interacts with intact biotin-labelled E-boxes (E2,E3,E4) but does not interact with mutated biotin labelled E-boxes (ΔE2,ΔE3, ΔE4) respectively. A protein-DNA complex shift is identified in intact Snai1 E-boxes (E2-E4) but this interaction is reduced when nuclear extracts are incubated with a 250-fold molar excess of unlabeled oligonucleotide probes. A supershift (black arrow) is identified in both E-boxes E3,E4 using 3ug Scleraxis AB ,while E2 we used 1 ug of scleraxis AB. Bands on (NS) lane is non-specific bands that are related to isoform of biotin-probe.(empty arrow) non-specific bands.

5. TGF-β1 mediated-Snai1 expression requires Scleraxis.

Our group has identified the role of TGF-β1 in inducing Scleraxis expression in primary cardiac fibroblasts through Smad-dependent and independent pathways [120, 121]. Moreover, the role of TGF-β1 extends beyond being a profibrotic cytokine as it also induces EMT in the human alveolar epithelial cell line, A549, this response is associated with Snai1 expression. When TGF-β1 is overexpressed in A549 cells, the epithelial cell line undergoes multiple morphological changes that begin to mimic a mesenchymal phenotype [135]. In general, this transition is initiated due to TGF-β1-mediated Snai1 expression, thus, these observations led us to investigate the role of Scleraxis in this process.

Our data demonstrate that Scleraxis is required for TGF-β1 mediated Snai1 gene expression. A549 cells were treated with shRNA targeting Scleraxis (AdshScx) or control (AdshLacZ), then treated with or without TGFβ1. TGFβ1 treatment results in a significant up-regulation of Snai1 expression when added with AdshLacZ control, thus, in the presence of Scleraxis protein. In contrast, Scleraxis knockdown reduced the effect of TGFβ1 on inducing Snai1 gene expression but was not able to completely diminish it. This indicates that Scleraxis is required for maximal TGFβ1-induced Snai1 expression in A549 epithelial cells (Fig. 9).

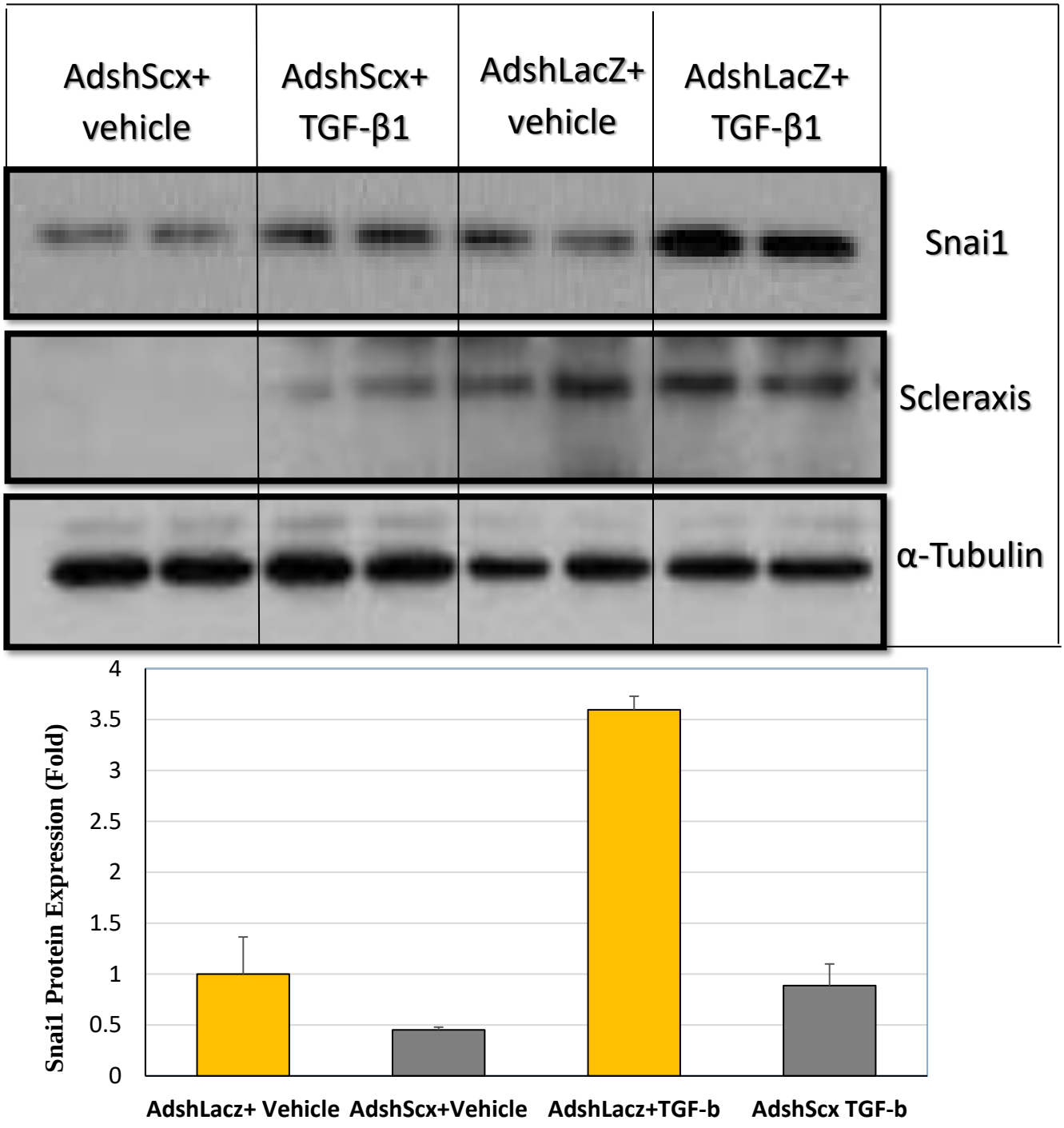

Fig. 9: Scleraxis is required for TGFβ1-mediated Snai1 expression

A549 human basal alveolar epithelial cells were infected with shRNA targeting Scleraxis (AdshScx) or LacZ control (AdshLacZ) for 48 hr, then additionally treated with either $TGF\beta_1$ (10 ng/ml) or vehicle for 24 h.

Total protein lysates were collected for western blotting, and the blot was probed for Snai1, Scleraxis and α-Tubulin as a loading control, n=2.

6. Scleraxis induces cell phenotype conversion and migration of A549 cells.

Scleraxis is a novel transcription factor that is known to regulate many ECM genes in cardiac fibroblasts and to induce cell phenotype conversion. Its upregulation in A549 cells was found to be critical in reducing epithelial markers while upregulating mesenchymal markers [125].

TGF-β1 is a potent inducer of EMT through up-regulation of Snai1 expression. Also, according to our recent finding, as mentioned previously, Scleraxis mediates the TGF-β1-mediated Snai1 expression. This led us to investigate whether Scleraxis induces responses that are consistent with EMT.

One of the key characteristics of EMT is that cells change their behavior to exhibit a more motile and migratory phenotype, which is a mesenchymal feature.

We therefore tested whether Scleraxis is able to induce A549 cells to migrate from their original epithelial sheet layer and become more motile. Wound heal assays (scratch assays) were performed using a confluent layer of A549 epithelial cells. These cells were treated with either adenovirus encoding Scleraxis (AdScx) or a control (AdGFP). 48 hours later, a scratch was performed in each plate of cells, which were then visualized after injury (0 hrs and 24 hrs).

Strikingly, control (AdGFP) and blank (non-infected) cells both had the same influx of cells, however, there was a higher migration rate in cells infected with AdScx (Fig. 10)

Interestingly, our findings are counter to the results of Scleraxis overexpression in cardiac fibroblasts by which cell migration is repressed [136]. This indicates that the *Scleraxis* gene has an integral role in cell phenotype conversion and this functional role is cell type-specific.

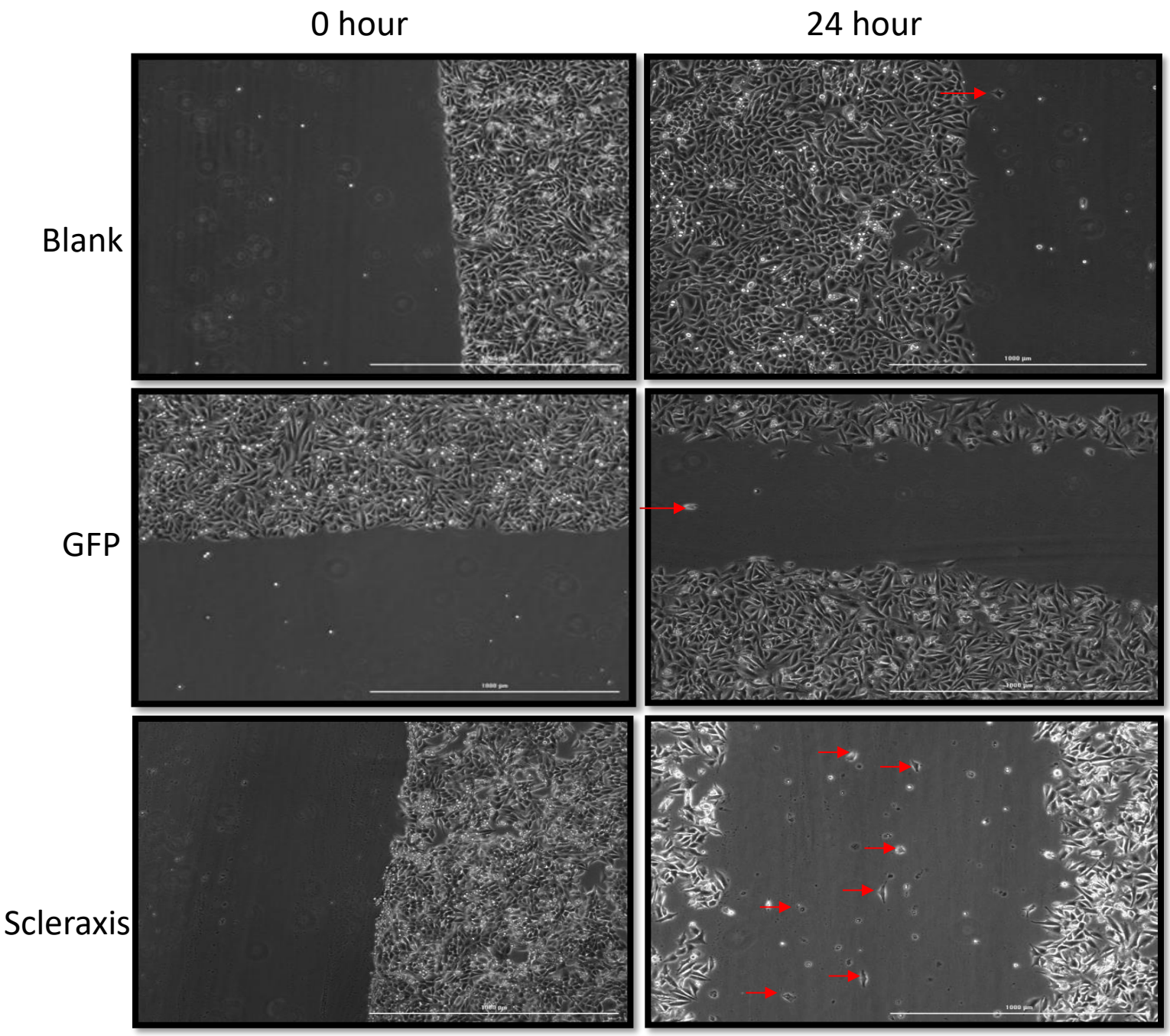

Fig. 10: Scleraxis induces migration of A549 cells

Cell imaging of basal alveolar epithelial A549 cells that were infected with Scleraxis-expressing adenovirus (AdScx ,100 MOI) or enhanced green fluorescent protein (AdGFP , MOI 100) as a control for 48 hours. Then a scratch was made across each of the confluent treated/untreated cells then images were taken at the time of scratch (0 hours) and 24 hours later. Cells migrated to the scratch area (red arrows-not all cells indicated). Using Cell Citation Imager, phase contrast images.

7. Scleraxis induces EMT by repression of E-Cadherin independent of Snai1 regulation.

Down-regulation of E-Cadherin is a critical step during EMT. Although the main down-regulator for E-Cadherin is Snai1, we wanted to examine if Scleraxis overexpression can repress E-Cadherin, in the absence of endogenous Snai1 in A549 cells.

Initially, we examined the efficacy of siRNA targeting Snai1 in attenuating Snai1 expression compared with scrambled siRNA. Therefore, we transfected A549 cells with either siRNA and analyzed our findings through Western blotting. We found that the efficacy of Snai1 siRNA is approximately 90%, indicating that Snai1 siRNA efficiently knockdown Snail in these cells (Fig. 11).

Using a 6-well plate, we knocked down Snai1 expression in A549 cells using Snai1 siRNA for 24hours, then induced Scleraxis expression by infecting the cells with AdScx for an additional 48 hours until cells were harvested, and lysates were analyzed by western blotting.

Our results verify that Scleraxis overexpression attenuates E-Cadherin expression in the absence of endogenous Snai1 expression. However, E-Cadherin could be further down-regulated in the presence of both Scleraxis and Snai1 compared to Scleraxis plus siRNA targeting Snail1, indicating that Scleraxis may act together with Snai1 in downregulating E-Cadherin expression and inducing processes consistent with EMT (Fig. 12).

This data indicates that Scleraxis is sufficient to attenuate E-Cadherin either indirectly through transactivating Snai1 expression, or directly through binding to E-Cadherin promoter, although further investigation of this possibility is needed.

In general, our findings confirm that Scleraxis is a central regulator of processes associated with EMT.

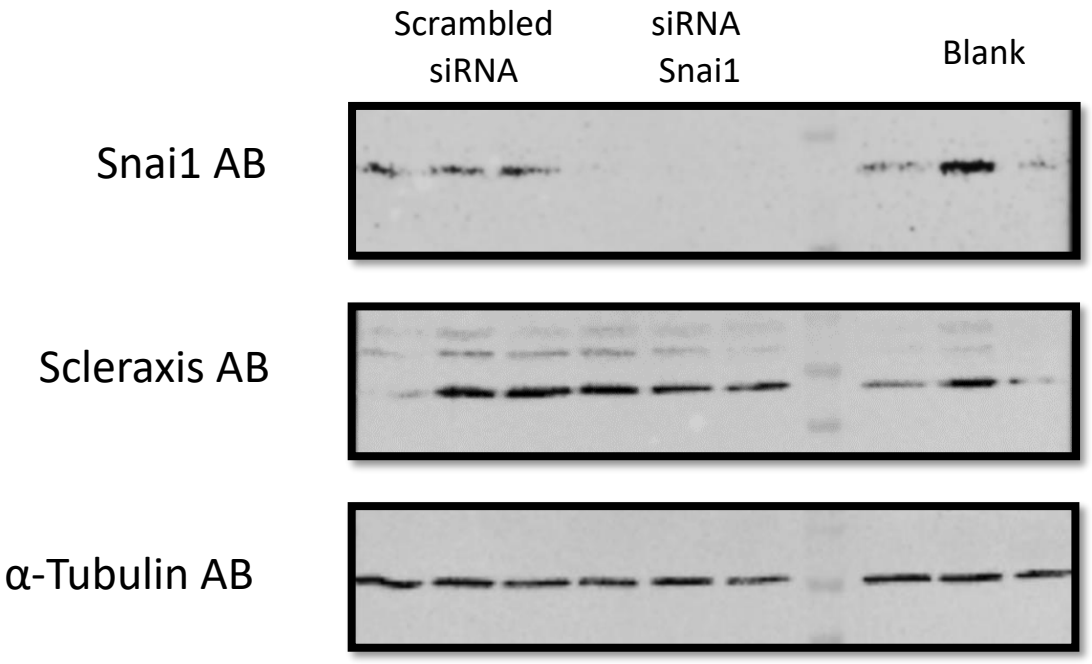

Fig. 11: Confirming the efficacy by which Snai1 siRNA attenuates the expression of Snai1 in A549 cells.

A549 cells were plated in 6-well plates and transfected with 50 pmol/well of either siRNA Snai1 , or scrambled siRNA or nothing added (blank). 48 hours later lysates were analyzed through western blotting. The blot was probed for Snai1, Scleraxis and α-tubulin as a loading control. siRNA targeting Snai1 showed a high efficacy in knocking down Snai1 expression in these cells.

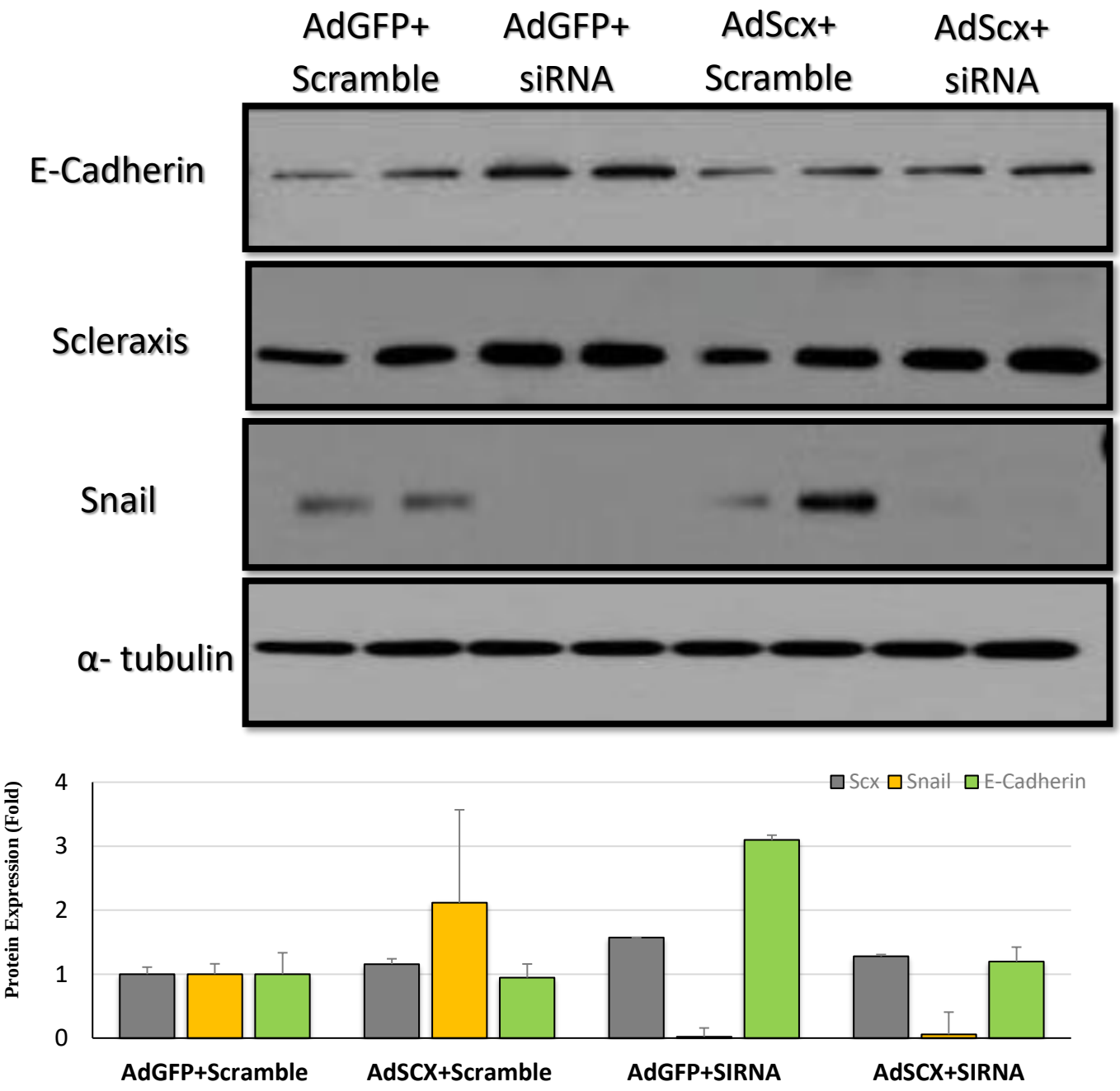

Fig. 12: Scleraxis downregulates E-Cadherin independent of Snai1 expression.
A549 epithelial cells were transfected with siRNA targeting snail1 (siRNA Snai1) or scrambled siRNA as a control for 24 h, then additionally they were infected with AdScx or AdGFP as a control for 48 h. Scleraxis overexpression alone shows an increase in both Scleraxis and Snai1 expression. SiRNA Snai1 shows knocking down of Snai1 while a 2-fold increase in E-Cadherin expression. The presence of Scleraxis expression along with Snai1 siRNA would attenuate the level of E-Cadherin less than when using Snai1 aiRNA alone. This indicates that Scleraxis is able to attenuate E-Cadherin expression independent to Snai1 expression. Total protein was collected for western blotting, then the blot was probed for E-Cadherin, Snai1, Scleraxis and α-tubulin as a loading control, and are representative of n=2 experiments. vs AdGFP+Scramble.

❖ Discussion

EMT is an integral process that occurs during embryonic development, fibrosis and cancer. It is characterized by a cell phenotype conversion that is regulated by many factors, including *Snai1* which represses the adhesion molecule E-Cadherin, thus facilitating their acquisition of a mechanical phenotype [66]. Our findings reveal that the Scleraxis transcription factor modulates processes associated with EMT in A549 cells, in part, through direct transactivation of Snai1 expression, which can promote EMT (fig .12). Our group has investigated the role of Scleraxis in cardiac fibrosis. We found that Scleraxis over-expression significantly increases expression of some important cardiac ECM genes such as type I collagen, and the contractile protein α-Smooth muscle actin (α-SMA). Conversely, loss of Scleraxis down-regulates these genes [125].

TGF-β1 is a well-known profibrotic factor associated with chronic inflammatory diseases in different organs. It can induce multiple ECM genes through Smad-dependent and Independent pathways [137]. It also induces Scleraxis gene expression by Smad-independent pathways in cardiac myofibroblasts [116, 120]. In combination, TGF-β1 and Scleraxis are important drivers of fibrosis, including cardiac fibrosis [125] . TGF-β1 also plays an important role in inducing EMT by upregulating Snai1 gene expression [56]. Our data confirms that Scleraxis is required for TGF-β1-mediated Snai1 expression and that Scleraxis induces processes associated with EMT.

Based on our new evidence, we suggest that matrix remodeling during cardiac fibrosis could include EMT, where EMT promotes accumulation of mesenchymal cells that expand the pool of fibroblasts available for phenotype conversion. The conversion of fibroblasts to myofibroblasts has also been described as a continuation of the EMT process that gives rise to fibroblasts in the myocardium [138]. However, further studies are needed to examine the exact correlation

between EMT- associated mechanisms and cardiac fibrosis. It is noteworthy that EMT may play a minimal role in cardiac fibrosis following MI, according to a recent paper by the Molkentin group, and the origin of cardiac myofibroblasts is primarily from cardiac resident fibroblasts [27]. However, it remains to be seen whether this finding applies to other forms of cardiac remodeling in the heart.

Cancer progression is mainly characterized by increased motility and migration of cells away from their origin. In EMT, epithelial cells lose their adhesion to the basement membrane and become more freely motile to penetrate tissues. Snai1 was found to contribute to many cancer cell lines including breast, lung, ovarian carcinomas [86-88]. Our data show that by overexpressing Scleraxis in A549 epithelial cells, cells exhibit more motile and migratory behaviour similar to metastatic behaviour during tumor progression. Although , there is no clear evidence that links between Scleraxis and cancer in literature, but our findings further supports the notion that Scleraxis could potentially induce cells to adopt metastatic features and contributes to cancer.

Scleraxis-induced fibrosis is not likely restricted to cardiac fibroblasts as it may extend to involve other tissues undergoing fibrosis such as in liver, or kidney. Scleraxis triggers multiple ECM genes and regulates phenotype conversion of FB`s to MFB`s in the heart to contribute to cardiac fibrosis [125]. Myofibroblasts are the major mediators of tissue fibrosis which are found in different organs including the lungs, skin, kidneys. For example, EMT play a prominent role in lung fibrosis [139]. Our studies were mainly performed on A549 alveolar basal epithelial cells that are derived from a lung cancer line. We show that Scleraxis transactivates Snai1 and is sufficient to repress E-Cadherin and knock down Snai1. This indicates that Scleraxis can induce EMT-associated signaling in the lungs causing pulmonary fibrosis. Overall, our findings suggest

that Scleraxis plays a significant role in EMT associated process, and may be a contributor to pathologic tissue remodeling and fibrosis.

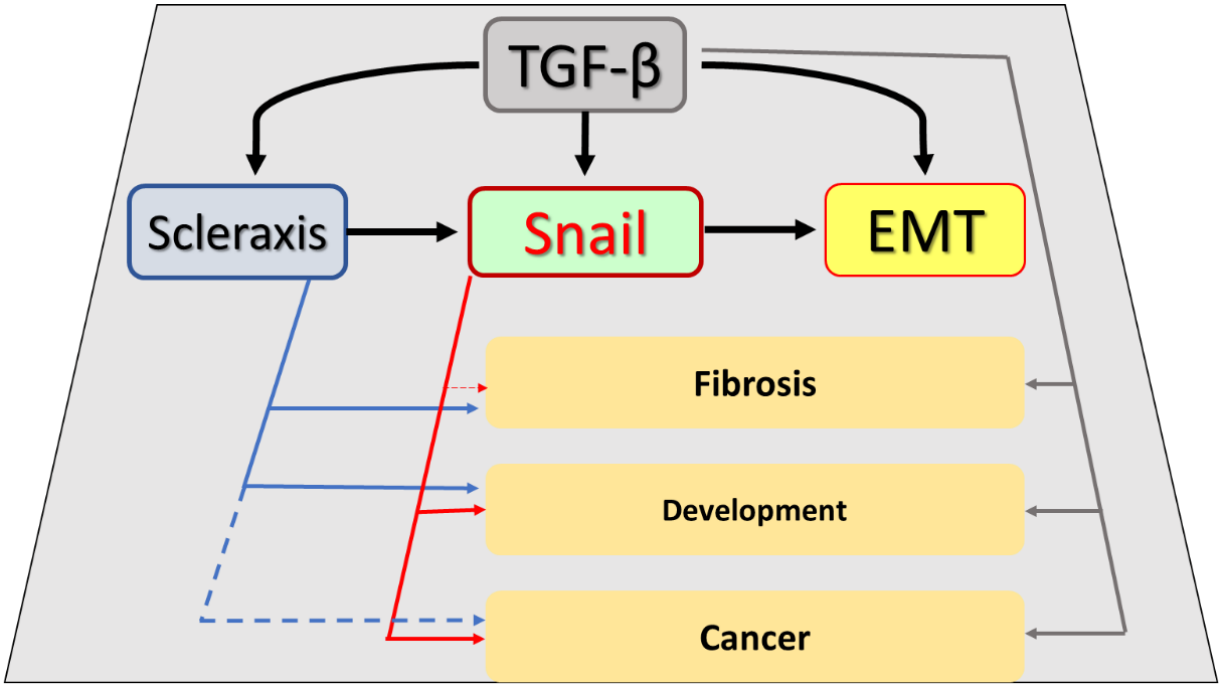

Fig. 13: Overall interlinked transcription factors that contribute to multiple EMT associated processes.

EMT with all its types (yellow boxes) is associated with multiple factors. Transforming growth factor beta1 (TGF-β1) induces Scleraxis and Snai1 gene expression to cause Snai1 up-regulation and contributes in all types of EMT (gray arrows), while Scleraxis transcription factor transactivates Snai1 expression and also contributes to EMT in fibrosis and development (blue arrows). Scleraxis could potentially contributes to EMT involved in Cancer (dashed arrow). Snai1 transcription factor contributes to all three types of EMT at various levels (red arrows) and directly induces EMT through E-Cadherin repression.

❖ Conclusions

Based on our data, we conclude that:

1. Scleraxis over-expression increases Snai1 expression in two different cell types.
2. Scleraxis directly regulates Snai1 expression by binding to at least three E-boxes on its promoter.
3. Scleraxis is sufficient for Snai1 transactivation and is required for maximal TGF-β1 mediated Snai1 gene expression.
4. Scleraxis induces morphological cell conversion and migration in A549 cells that is consistent with EMT.
5. Scleraxis could be a potential inducer for cell responses associated with EMT.

❖ Significance

In response to an injury or an inflammatory process, fibrosis usually follows as fibroblasts become activated and converted to myofibroblasts producing excessive amounts of extracellular matrix. Initially, this mechanism occurs to protect and repair the organ from further functional instability causing fibrosis. Normally, this scar is gradually degraded, however, in certain organs this fibrosis persists, impeding the physiological function of the organ and causing further insult. Our goal is to be able to control this process through interfering with the specific factors along the pathway such as Snai1 or Scleraxis.

Therefore, considering that fibrosis is associated with EMT, our lab has investigated the role of Scleraxis in transactivating multiple ECM genes and inducing fibroblast phenotype conversion. Here, we have shown that Scleraxis binds to Snai1 and induces EMT. Thus, we believe that interfering with the Scleraxis-Snai1 axis could alter the EMT process and attenuate fibrotic progression in different diseases.

EMT is a broad-spectrum cell response that needs to be investigated further, having been highly associated with metastasis, tumor progression and cancers. Multiple factors contribute to this type of EMT; most apparently, *Snai1* gene expression is considered a poor prognostic marker for metastasis. As we have determined that Scleraxis controls Snai1 expression, it would be of importance to investigate whether Scleraxis plays a role in cancer and tumor progression.

Our findings have revealed the link between Scleraxis and some EMT-associated pathways, and specific interference of the Scleraxis axis to alter EMT processes could be a target for a number of diseases.

❖ Limitations of the study

In this study, the use of the human lung carcinoma epithelial cell line (A549 cells) was to emphasize the effect of Scleraxis in promoting EMT, and treating them to observe their behavior. A549 cells are a good model to examine the processes inducing EMT and to measure epithelial and mesenchymal markers. However, this doesn't exclude the possibility that Scleraxis has different effects in epithelial cells from different organs.

Our experiments also involved multiple cell lines (NIH 3T3, Human cardiac fibroblasts, Hek293), each for different experiments and purposes. For example, NIH3T3 is a fibroblast cell line that is inexpensive and easier to manage and transfect using our luciferase promoters in combination with other vectors in luciferase reporter assays. We also used human cardiac fibroblasts in the ChIP assay, and confirmed a similar mechanism of Scleraxis binding to the Snai1 gene promoter. Hek293 cells were used to obtain nuclear lysates for EMSA. They are very easy to transfect, and generate higher levels of proteins as they tend to grow at a larger scale. Although we used multiple cell lines, it is noteworthy that similar results were obtained regardless of cell type, suggesting that the mechanisms we describe are universal. It is worth mentioning that using variable cell lines *in vitro* to evaluate the mechanism of Scleraxis regulation and the outcomes on EMT are limited, and our results need to be examined *in vivo*.

Further studies are required, using different cell models with an adequate number of experiments to be able to verify the exact mechanism by which Scleraxis-Snai1 axis is involved in EMT, and the extent of its contribution in the pathological processes related to EMT- induced diseases.

❖ Future Directions

Understanding the mechanism by which Scleraxis transactivates Snai1 expression and thus induces EMT builds a basic molecular interaction pathway that could contribute to a broader understanding of fibrosis and cancer. However, understanding whether Scleraxis can promote EMT through interaction with and down-regulation of the E-Cadherin gene needs further investigation. Also, it is important to verify if Scleraxis down-regulation inhibits the EMT-associated pathways.

Interestingly, E-Cadherin down-regulation can be triggered through the action of E12/E47 during development and tumor progression [64]. Both E12 and E47 are class A bHLH proteins that are known to form heterodimers with Scleraxis, where it synergizes the affinity of Scleraxis binding to E-boxes [106]. It will be important thus to examine if this dimerization has an additive effect in inducing EMT-associated pathways.

In addition, in linking Scleraxis to EMT, and since Scleraxis is a potent regulator of fibrosis and plays a significant role in embryogenesis, it would also be interesting to test the role of Scleraxis expression in tumor initiation and progression.

Further studies and assessments need to be investigated to involve a broader direction of pathogenesis and cell phenotype conversion. Understanding the basic mechanism underlying diseases involving Scleraxis-Snai1-E-Cadherin axis would provide us a potential target to limit undesirable EMT-mediated pathology.

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