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A MECHANISM FOR THE FGF2-MEDIATED DOWN-REGULATION OF
INTEGRIN ALPHA-11 IDENTIFIED THROUGH STUDYING THE ALTERED
ADHESOME OF HUMAN DERMAL FIBROBLASTS UNDERGOING EARLY
MESENCHYMAL-TO-EPITHELIAL TRANSITION

A Dissertation

Submitted to the Faculty

of

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In Partial Fulfillment of the Requirements for the Degree of

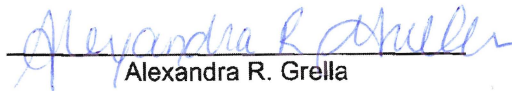
Doctor of Philosophy

In

Biology and Biotechnology

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by



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A mechanism for the FGF2-mediated down-regulation of integrin alpha-11 identified through studying altered adhesion of human dermal fibroblasts undergoing early Mesenchymal-to-Epithelial Transition

Abstract

Work in our lab has resulted in the development of a novel approach to creating a more developmentally plastic human dermal fibroblast (hDF) phenotype that allows for the study of molecular mechanisms involved in cell-fate conversion. Culturing hDF under defined culture conditions (5% O₂ and supplementation with fibroblast growth factor FGF2) induces the regeneration competent (iRC) phenotype that is characterized by stem cell gene expression, and increased life-span *in vitro*. The work presented in this thesis further characterizes the system, and describes an overall shift in extracellular matrix and adhesion molecules in human dermal fibroblasts (hDF) undergoing the transition to a more developmentally plastic phenotype (iRC). This work suggests that we create the initiation phase of Mesenchymal-to-Epithelial Transition (MET) during conversion to the iRC phenotype. This transition is marked by loss of integrin alpha-11 (α 11) and its binding partner Collagen-I (COL-I). Moreover, we describe the mechanism for the down-regulation of α 11 that is mediated by FGF2 activation of ERK1/2 through systematic investigation of several potential molecular mechanisms. The body of work presented here shows that the ERK 1/2 mediated down-regulation of α 11 is independent of activation of TGF- β 1-mediated regulation of α 11. In addition to down-regulation of α 11, an overall shift in the transcript levels of other adhesion molecules is observed, which demonstrates that iRC are most likely transitioning their attachment to a laminin and fibronectin-based matrix. These results suggest that iRC may be producing a more “pro-regenerative matrix”. We hypothesize that the changes in integrin expression profile and interaction with ECM serve as a feedback loop during the iRC phenotype shift. Our findings suggest that this “pro-regenerative” shift in attachment of iRC as well as the ERK 1/2 mediated down-regulation of α 11 could be exploited in wound healing biology and fibrosis research. Manipulation of the dynamic relationship between TGF- β 1 and FGF2 has the potential to reduce scar deposition. Further identification of molecular mechanisms controlling this phenotype conversion will allow development of strategies for *in situ* manipulation of wound healing outcomes.

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Abbreviations

2D: Two dimensional
3D: Three dimensional
AKT: protein kinase B
AM: Adhesion molecules
ANOVA: Analysis of variance
BSA: Bovine serum albumin
cDNA: Complementary DNA
COL-1: Collagen type-1
CpG: Cytosine-phosphate-guanine
CSC: Cancer stem cell
DAB: 3,3'-Diaminobenzidine
dATP: Deoxyadenosine triphosphate
DMEM: Dulbecco's modified eagle's medium
DNA: Deoxyribonucleic acid
ECM: Extracellular matrix
EMSA: Electromobility shift assay
EMT: Epithelial to mesenchymal transition
ERK1/2: Extracellular signal regulated kinases
ESC: Embryonic Stem Cell
F12: Ham's F12
FAK: Focal Adhesion Kinase
FBS: Fetal bovine serum
FCS: Fetal calf serum
FGF2: Fibroblast growth factor 2
FGFR: Fibroblast growth factor receptor
GFP: Green fluorescent protein
HCL: Hydrochloric acid
hDF: Human dermal fibroblast
hESC: Human embryonic stem cell
HMW: High molecular weight
HSPG: Heparin sulfate proteoglycan
ICC: immunocytochemistry
IFN: interferon
IMAC: Immobilized metal affinity chromatography
iPSC: Induced pluripotent stem cells
iRC: Induced regeneration competent cell
ITGA11: Integrin alpha-11
JNK: Jun amino-terminal kinases
LMW: Low molecular weight
MAPK: Mitogen activated protein kinase
MDE: Methylation dependent enzyme
MEF: Mouse embryonic feeder
MET: Mesenchymal to epithelial transition
MIDAS: metal ion-dependent adhesion site motif
miRNA: microRNA
MMP1: Matrix metalloproteinase-1

MMPs: Matrix metalloproteinases
MRNA: Messenger ribonucleic acid
MSE: Methylation sensitive enzyme
PAGE: Polyacrylamide gel electrophoresis
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PD: Population doubling
PI3K: phosphoinositide 3-kinase
PLCY: Phospholipase C γ
PVDF: Polyvinylidene difluoride
qRT-PCR: Quantitative RT-PCR
RNAi: RNA interference
RPE: Retinal pigmented epithelial cell
RT-PCR: reverse transcription polymerase chain reaction
SCID: Severe Combined Immunodeficiency
SD: Standard deviation
SDS: Sodium dodecyl sulphate
Sp1: Specificity protein 1
TAE: Tris acetate EDTA
TBST: Tris buffered saline tween
TGF- β 1: Transforming growth factor β 1

Chapter 1

Background and Literature Review

1.0 Introduction

Discoveries that have enabled the conversion of mammalian somatic cells towards a less specialized cell type with higher developmental potential hold great therapeutic potential encompassing a broad range of applications. Induced acquisition of multipotency or pluripotency not only allows for generation of an autologous cell source and patient-specific disease modeling, it also creates the opportunity to address a diverse range of developmental biological questions that could not be studied previously due to unavailability of suitable models.

We have developed a novel approach to creating a more developmentally plastic human dermal fibroblasts (hDF) phenotype that allows us to study the molecular mechanisms involved in cell-fate conversion. Culturing hDF under defined culture conditions (5% O₂ and supplementation with fibroblast growth factor FGF2) induces stem cell gene expression, and increases their life-span *in vitro*. The iRC cells maintain a normal karyotype and do not form tumors when injected into severe combined immunodeficiency – SCID - animals. The developmental plasticity of cells cultured under these conditions has led us to call them induced regeneration competent cells (iRC). This plasticity has been demonstrated by transplantation into a murine volumetric skeletal muscle wound where they engraft, acquire expression of a muscle satellite stem cell marker PAX7, and instead of significant collagen deposition and scar formation contribute to wound healing by functional regeneration of skeletal muscle [1].

The ability of the iRC's to profoundly alter the outcome of a wound healing process from scar formation to regeneration prompted the work presented in this thesis, namely to investigate the role of low oxygen and FGF2 culture system in regulation of extracellular matrix (ECM) and adhesion molecules (AM) that may be involved in the fibroblast phenotype conversion.

1.1 Regenerative Medicine—Eliciting functional regeneration as a response tissue damage

Regenerative medicine dates back to early 3rd century writings. For as long as we have written records, it is clear that doctors and scientists alike have been aware of the need for regenerative medicine as a means to replace damaged or diseased parts of the body. Regenerative medicine has developed in parallel with modern medicine, with the most critical landmark being in the field of tissue engineering, when Johann Friedrich Dieffenbach in 1882 performed the first skin transplantation in animal models [2].

A major development that significantly affected the field of regenerative medicine was the ability to grow human embryonic stem cells *in vitro* [3]. This discovery marked an important milestone not only in the stem cell biology field, but in clinical research and personalized medicine as well [3]. Groundbreaking work in the field of reproductive biology leading to the development of somatic cell nuclear transfer technology [4] changed the way clinicians and scientists viewed the potential for regenerative medicine.

1.1.1 Degenerative diseases

Human diseases that can be treated by the stem cell-based therapies are a diverse, broad range of diseases. Stem-cell based therapy have potential applications in treating hematopoietic disorders, ranging from cancers such as leukemia and myeloma, to various forms of anemia related disorders [5]. Stem-cell based therapies offer hope for neurological degenerative disorders such as Parkinson's and Alzheimer's, as well as spinal cord injury and multiple sclerosis [6]. They have also been implicated to as potential therapies for diabetes mellitus as well as a broad range of other degenerative disorders of the eyes, liver, lungs, and skin.

Animal studies using embryonic stem cells differentiated into photoreceptors *in vitro* and transplanted into blind animals showed good integration [7], and currently similar technology is

being used in clinical trials where retinal pigmented epithelial (RPE) cells are currently being derived from human embryonic stem cells [8] and transplanted into damaged cornea of patients for treatment of dry age-related macular degeneration. This is the first therapy in humans to demonstrate efficacy using human embryonic stem cells. Early results from these clinical studies have shown that human embryonic-derived RPE cells can repair and restore vision in patients [9-11].

Recent studies aiming at the use of stem cells for treating myocardial infarction have gained importance, and animal studies have demonstrated very positive outcomes for treatment of myocardial infarctions with stem cells or stem cell-derived cardiovascular cells. Several studies in patients suffering from acute myocardial infarction are exploring the use of autologous bone marrow stem cells or hematopoietic stem cells in combination with current pharmacological and surgical interventions. Early results from these studies are very encouraging and have demonstrated improvements over standard approaches [12, 13].

1.1.2 Injury and Wound Healing

Skeletal muscle has a large potential for regeneration following injury, however few effective therapeutic options exist for traumatic injury resulting in volumetric muscle loss. When more than 20% of the muscle tissue is lost, injury results in the natural repair process failing, and accumulation of scar tissue, and damage to the surrounding muscle tissue [14-18]. Autologous muscle grafts or muscle transposition represent two possibilities of salvaging procedures for the restoration of tissue function, but at best they have limited success [19]. Most cell-based therapies are still in their infancy, and a large need exists for regenerative strategies based in cell therapy applications for regeneration of skeletal muscle after volumetric muscle loss. Most work that has been done in repairing muscle functionality using stem cell-based therapies has been treating degenerative diseases such as muscular dystrophies [20-24]. There is still a need for a translational application for the isolation, expansion and maintenance

of myogenic cells from an autologous cell source for the treatment of volumetric muscle loss, as it requires a larger cell source than the treatment of degenerative diseases [25] .

The ultimate goal of the treatment of cutaneous burns and wounds is to restore the damaged skin both structurally and functionally to its original state. Recent research advances have shown the great potential of stem cells in improving the rate and quality of wound healing and regenerating the skin and its appendages. Stem cell-based therapeutic strategies offer new prospects in the medical technology for burns and wounds care. With regard to its therapeutic implication in skin, an earlier preclinical study has demonstrated that human ESCs can be differentiated into fully functional keratinocytes and thus can be used to grow epidermis *in vitro* and provide temporary skin substitutes for patients awaiting autologous skin grafts [26]. In a recent study, it was validated that iPSC's could be derived from fibroblasts of patients with an inherited genetic disease characterized by recurrent blistering skin wounds. Moreover they showed that these iPSCs were able to generate 3D skin equivalents *in vitro* suggesting functionality [27]. The validation of this iPSC therapy proves that iPSC can be a source for the generation of keratinocytes for stem cells for wound healing applications.

Stem cells have been utilized in a few regenerative skin applications. Reports published in 2005 described healing of an extensive skin burn after transplantation of bone marrow derived mesenchymal stem cells [28]. There is a need for treatment methods for patients with extensive skin burns, which would be lifesaving, but engineered skin is not structurally and functionally equivalent to normal skin and so ongoing studies are exploring whether iPSC's could be used to bridge this gap. iPSCs derived from different cell types of the skin could then be combined and differentiated into all the cell types of the skin allowing a more effective regeneration of damaged skin [29].

1.2 Regenerative Medicine—Search for a cell source

1.2.1 Pluripotent Cell Sources

Human embryonic stem cells (hESC) are derived from the inner cell mass of a blastocyst and *in vitro* can serve as a self-renewing source of pluripotent cells. Pluripotent cell sources offer the ability to differentiate into any of the three germ layers: endoderm, mesoderm and ectoderm [30]. The development and study of methods for culturing hESC created a path to advancements in regenerative medicine. In addition to their therapeutic potential, hESC offer the ability to study unique biological systems and disease models that were very difficult to reproduce prior to hESC culture development.

Human embryonic stem cells *in vitro* grow in colonies that are composed of highly adherent pluripotent progenitors, where pluripotency is maintained through a defined culture system with a large component being the presence of a mouse embryonic feeder layer, or a combination of purified extracellular matrix substrates [31]. Dissociation of cell-cell attachments in hESC colonies leads to an increased rate of differentiation, and cell death. This need for attachment both by extracellular contact and cell-cell contact suggest that both substrate and cell-cell adhesion are seminally involved in the assembly and maintenance of pluripotent human embryonic stem cell colonies [32].

The large number of benefits and the future potential that hESCs provide for the regenerative biology community and the field of drug discovery research are met with a list of ethical problems and limitations. As a result of these limitations, large research efforts were focused in developing pluripotent stem cells from other sources. These research efforts resulted in the discovery of induced pluripotent stem cells (iPSC).

iPSCs were originally generated by use of viral vectors, which introduced four key reprogramming factors (OCT3/4 and SOX2, with either a combination of KLF4 and C-MYC or NANOG and LIN28) into skin fibroblasts [33, 34]. Addition of these reprogramming factors

induces an embryonic-like state where cells can be passaged in culture indefinitely while retaining their pluripotency, or be differentiated into one of the three germ layers through varying media compositions, presence of cytokines, and extracellular matrix substrate [33, 34].

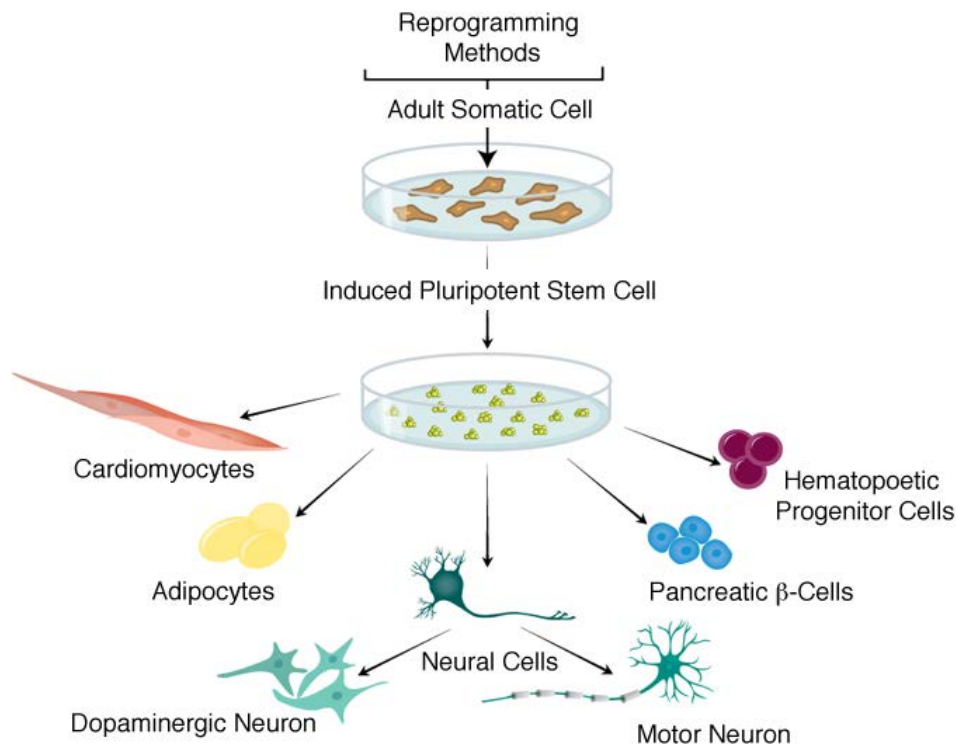


Figure 1.1 Schematic of iPSC induction

Adult somatic cells are reprogrammed through various methods to give rise to induced pluripotent stem cells which can be further differentiated into various cell types for therapeutic application and drug screening.

Regenerative medicine has given increased attention to the potential of iPSC in recent years. Potential therapies and technologies using cells that have been reprogrammed to an alternate fate could provide immune-matched cells for transplantation therapy, advancements in drug development, generation of a multitude of cell types for drug compound screening, and the

ability to study disease pathology through cell lines which are reproducible, and definable (Figure 1.1). However the successful implementation of personalized therapies employing iPSC technology depends on the generation of cells that are free of all safety and ethical concerns. As a method of direct reprogramming by defined-factors, iPSCs hold the potential for forward progress in regenerative medicine. However, to date, major roadblocks exist which limit the use of iPSC from derivation sources that are available [35, 36].

Much work is needed before cell lines derived from iPSC can their reach full therapeutic potential in personalized cellular therapy. Some progress has been made at varying success rates in the derivation of cell lines from iPSC through cell type specific differentiation protocols. Most differentiation protocols involve successful differentiation of hESCs and iPSC into functional hepatocytes both *in vitro* and *in vivo* [37-39], lung epithelium [40, 41] insulin secreting cells [42, 43], functional neurons [44, 45] as a treatment for many neurodegenerative diseases, and potentially restore injury from damage to the spinal cord (Figure 1.2).

To this end, progress towards defining novel methods for generation of a clinically complaint, autologous cell source is necessary. Moreover, understanding the molecular mechanisms underlying cell-fate and cell-fate reversal is critically important to the field of regenerative medicine for therapeutic advancement utilizing pluripotent cell lines as therapies. In addition to attachment for maintaining pluripotency, human embryonic stem cells are dependent on the addition of basic fibroblast growth factor 2 (FGF2) from either exogenous supplementation, mouse embryonic fibroblast conditioned culture medium, or a combination of the two [46, 47]. Recently, the methods for derivation and maintenance of stem cells have shifted focus to the importance of the environmental cues, with emphasis on the role of extracellular matrix (ECM) and adhesion molecules (AM) [48].

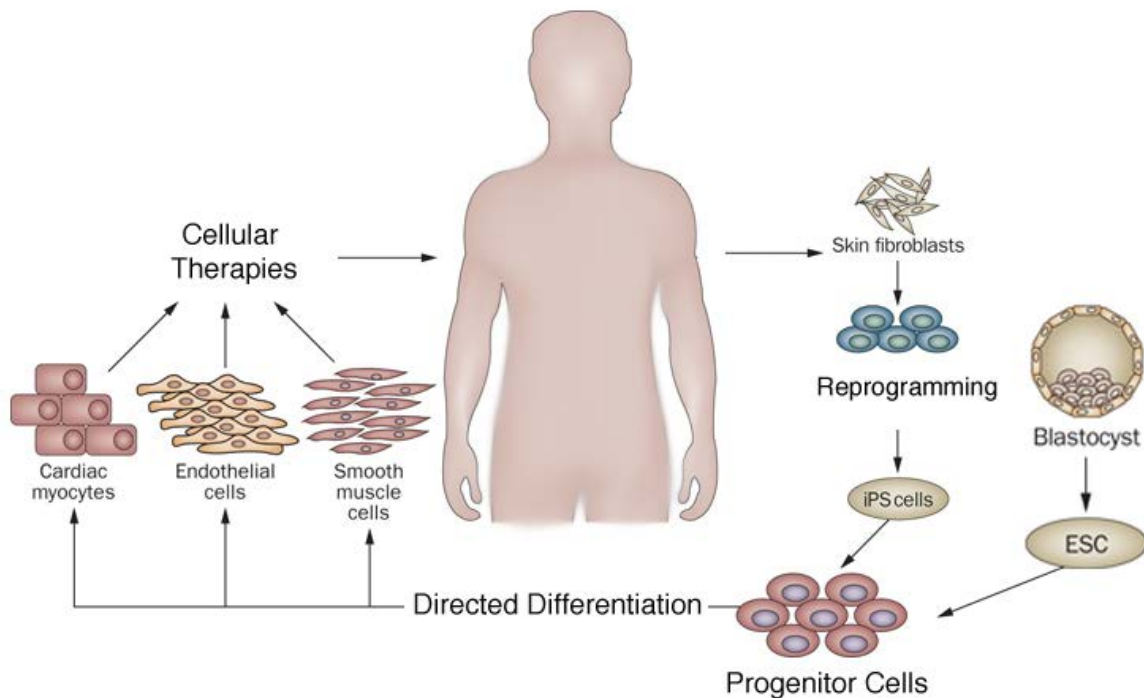


Figure 1.2 iPSC uses for cellular therapy

iPSC as autologous cell therapy through isolation of skin fibroblasts, reprogramming into induced pluripotent stem cells, and then differentiated into cell lineages useful for therapeutic application.

1.2.2 Multipotent cell sources-

Adult stem cells have become an attractive source for cell therapy as they have are developmentally plastic; they have the ability to become many cell types when given the right extracellular cues. Adult organs are mostly comprised of differentiated cells, however quiescent progenitor stem cells that have biological roles in physiological and pathological regeneration are found in discrete locations, or in systemic circulation. Identification of committed stem or progenitor cells has been done from various tissue sources, some of which include hematopoietic stem cells present in bone marrow [49], central nervous system stem cells from brain [50] ,skeletal muscle satellite cells [51] , and connective tissue mesenchymal stem cells [52]. Adult stem cells regenerate tissue by generating new populations of cells from progenitors.

Several experiments showing the differentiation potential for adult stem cell populations have given rise to increased interest in their use in therapeutic applications. With their potential also comes controversy, as they have limitations such as reaching replicative senescence [53],

they vary in differentiation potential based on the derivation source [52] and in some cases have been demonstrated an inability incorporate into host tissues [54].

1.2.3 *Trans-differentiated adult somatic cells- crossing lineage boundaries*

The direct reprogramming of one somatic cell to another somatic cell type is known as trans-differentiation. This has been demonstrated in a limited number of cases, with varying success rates. The general principle was founded out of iPSC work where it was hypothesized that if a fibroblast could be reprogrammed to a pluripotent cell, it may be able to cross lineage boundaries by forced transcription factor expression. It has been demonstrated that by either addition or ablation of specific transcription factors, in combination with cytokines and growth conditions, somatic cell sources can cross lineages into other somatic cell types. Fibroblasts have been shown to be directly convert to neurons by expressing neuronal lineage specific transcription factors *in vitro* [55]. Fibroblasts directly reprogrammed into blood progenitors by expression of hematopoietic transcription factors and a cocktail of cytokines have been shown to have functional engraftment *in vitro* [56]. While this approach holds potential, the need for forced transgene expression still harbors the same limitations as iPSC technology, and directly limits the clinical relevance of these trans-differentiated cell sources.

1.2.4 *Differentiated somatic cells – connective tissue fibroblasts*

The forced expression of genes to create cells for therapeutic purposes results in cells that are not therapeutically relevant. Therefore, identification of cell culture conditions, which are able to induce endogenous expression of these genes required for either iPSC or trans-differentiation, is critical for cell therapy. It has been demonstrated that fibroblasts have basal expression of mRNAs for stem cell genes, however the translation of these messages into properly localized proteins can be achieved by manipulation of oxygen concentration and FGF2 supplementation. This change in culture conditions was shown to induce expression of REX1, potentiate the expression of LIN28 transcriptionally, and translationally induce expression of

nuclear OCT4, SOX2, and NANOG. Culturing human dermal fibroblasts under these defined conditions increased the lifespan *in vitro*, however these cells were not truly pluripotent, as they never formed tumors in SCID mice [57].

1.3 Regenerative Medicine—Search for a mechanism

After the initial demonstration that somatic cells could be reprogrammed to pluripotency by defined factors [33, 34] studying the molecular mechanism of turning a somatic cell into a pluripotent cell was of high interest. Global studies looked at changes in the epigenetic state, as well as transcriptional changes throughout the reprogramming process.

1.3.1 Mesenchymal-to-Epithelial Transition

The first data to start to define this transition was a microarray data set, which looked at various time points within the reprogramming of MEFS [58]. This study showed that the immediate response to reprogramming factors was increased proliferation [58].

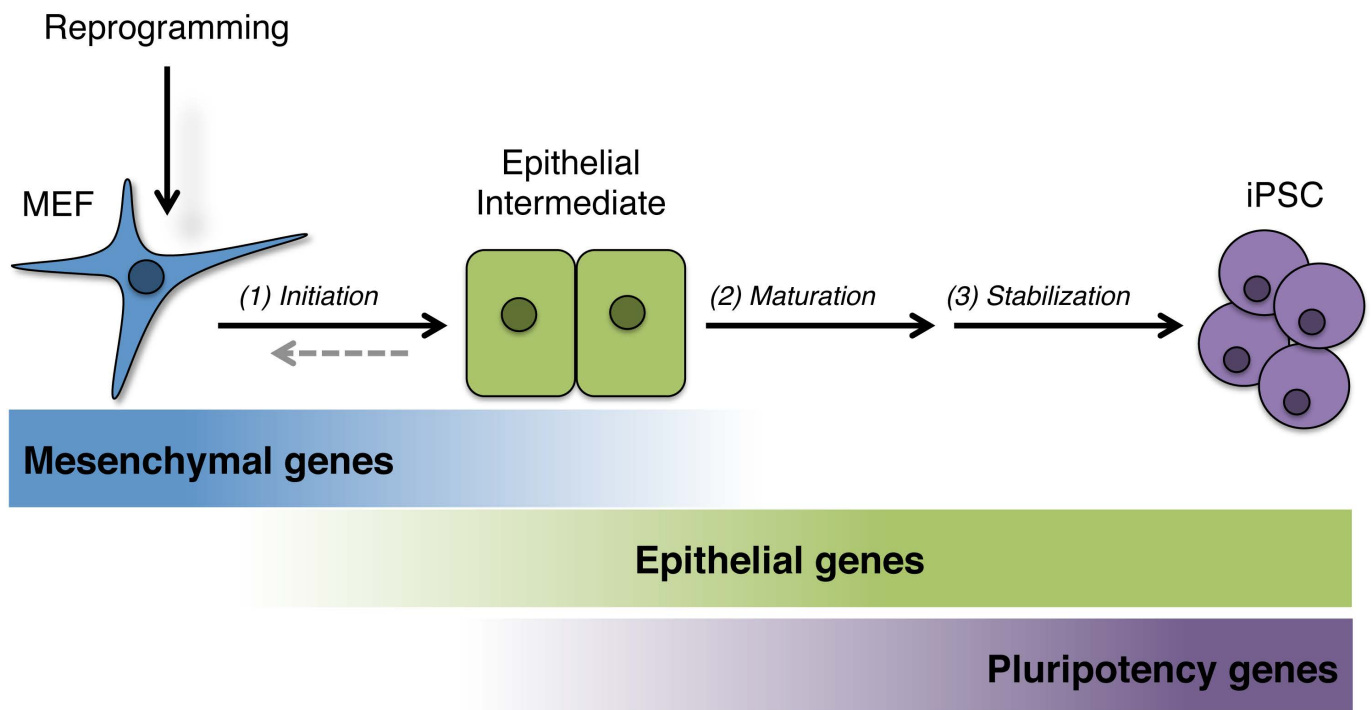


Figure 1.3 Mesenchymal-to-Epithelial Transition required for Reprogramming

When studying either gene expression profiles or morphological changes of MEFs during the initial stages of reprogramming to iPSC there were similarities to MET. This pattern included the up-regulation of epithelial genes as well as the down-regulation of key mesenchymal genes. Based on the observed gene expression dynamics three sequential phases of reprogramming were identified [58].

Subsequent microarray data, looking at the RNA-seq as well as RNAi screens, identified that fibroblasts undergoing reprogramming by defined factors go through three phases of reprogramming. These phases were termed by *Li, et al.* as (1) *initiation*, (2) *maturation* and (3) *stabilization*. Molecular events, which were identified and separated into these three phases, identified that a mesenchymal-to-epithelial transition was necessary within the initiation phase for cells to undergo reprogramming to a de-differentiated, induced pluripotent state [59, 60]. When studying late maturation phases of iPSC, it should be noted that transgenes required to drive reprogramming initiating events, and MET events are no longer necessary, however it is hypothesized that this is because genes involved in gametogenesis were up-regulated [61] (Figure 1.3). MET is dependent on expression of pluripotency transcription factors, and has been identified as the initiation step in acquisition of pluripotency through inhibition studies resulting in failure to induce pluripotency [59, 62].

In order to understand the functional requirement for MET in iPSC induction, the process by which MET and EMT occur in development is critical. The epithelial-to-mesenchymal transition (EMT), and the reverse mesenchymal-to-epithelial transition (MET), have central roles in embryogenesis [63] (Figure 1.4). Most adult tissues and organs are formed through a series of a highly organized conversions of epithelial cells to mesenchymal cells through EMT and MET.

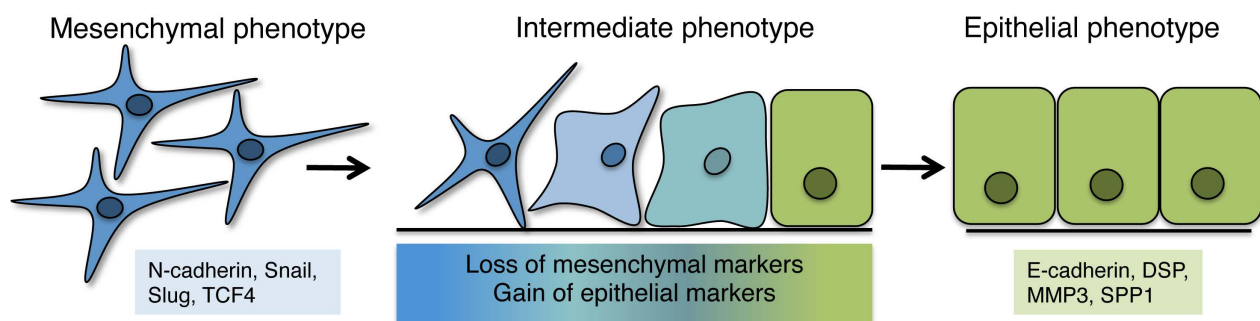


Figure 1.4 Mesenchymal-to-Epithelial Transition

Mesenchymal-to-Epithelial Transition (MET) is marked by the transition of a mobile mesenchymal cell to a polarized epithelial cell. The above schematic shows the transition from a mesenchymal phenotype to an epithelial phenotype via MET. Transition is marked by loss of mesenchymal markers and gain of epithelial markers.

Most critically, epithelial and mesenchymal cell phenotypes are reversible, and during embryonic development, cells can convert between the epithelial and mesenchymal states. EMT and MET conceptually were first defined by Elizabeth Hay 40 years ago [64]. Using the chick primitive streak as a model, Hay observed that epithelial cells were able to undergo a phenotypic changes that reflected a transformation to a mesenchymal cell (EMT) [64]. These events were subsequently observed in the reverse order (MET) [65, 66]. For the purposes of understanding the molecular events involved in cell phenotype conversion, it is necessary to understand MET.

Mesenchymal cells are loosely organized in a three-dimensional extracellular matrix, and comprise connective tissues adjacent to epithelia. The conversion of mesenchymal cells to epithelial cells is observed in embryonic development and involves phenotypic changes defined by the acquisition of cell-cell adhesion and cell-cell polarity and the loss of migratory phenotype. In early embryonic development, mesoderm is generated by EMT, which forms multiple developmental tissue types. Examples include mesodermal cells generating epithelial organs, such as the kidneys and ovary through a series of MET [67].

1.3.2 FGF2: a diverse potent signaling molecule

FGF2 is a vital growth factor involved in stem cell determination *in vitro* [47, 68]. In humans, FGF2 is produced as five isoforms (18, 22, 22.5, 24 and 34 kDa), which have different subcellular localizations and contribute to different cellular behaviors [69, 70]. FGF2 isoforms are generated by alternative initiation of translation of the same FGF2 mRNA [71]. The 18 kDa FGF2, termed low-molecular weight (LMW), is translated from a conventional Kozak [72] AUG start codon and consists of 155 amino acids, which represents the core sequence common to all FGF2 isoforms [73]. The additional four FGF2 isoforms are known as high-molecular weight isoforms, and are initiated by upstream CUG sites [74, 75]. LMW FGF2 signaling through FGF receptors (FGFRs) has roles in development in the adult such as, tissue repair, angiogenesis,

wound healing, and inflammatory response [69]. FGFR pathway disruption has been linked to many disease pathologies, including cancer [76, 77].

FGFRs are receptor tyrosine kinases structurally composed of an extracellular ligand-binding domain linked by a single transmembrane domain to the intracellular protein. FGFRs are activated in a ligand-dependent manner leading to receptor dimerization [78] that results in activation of the kinase domain, and signaling through the mitogen-activated protein kinase (MAPK) pathway. Dimerization and activation of FGFRs can also activate PI3K, PLC γ and signal transducers and activators of transcription. Examples of downstream effects of FGF2 signaling include proliferation, growth, differentiation, migration, and survival [69, 79].

The predominant signaling pathway activated downstream of FGFRs is MAPK signaling which has roles in both development [80] as well as cell proliferation by promoting cells to enter S-phase through activating expression of cyclin-D1 [81]. MAP kinases are a family of proteins that have a variety of cellular roles: ERK1/2 is known to be a regulation of mitosis; JNK1/2/3 is known to be involved in stress-activated inhibition of protein synthesis; and p38 $\alpha/\beta/\gamma/\delta$ are known to regulate expression of inflammatory cytokines [82].

1.3.3 Stem Cell Niche as a requirement for maintenance of potency

In order to maintain potency, human embryonic stem cells are dependent on growth in colonies which are highly adherent [31] and in addition to attachment, human embryonic stem cells are dependent on the addition of basic fibroblast growth factor 2 (FGF2), from either exogenous supplementation, or mouse embryonic fibroblast conditioned culture medium [46, 47].

Just as attachment of hESC is known to play a role in maintenance of pluripotency, a specialized niche is important for phenotype maintenance of adult stem cells. Adult stem cells are responsible for the growth, maintenance and overall homeostasis of most adult tissue types within the body. The survival and maintenance of multipotency is regulated by the

microenvironment in which they reside [83]. This microenvironment, otherwise known as niche, is defined as an anatomical compartment which signals to the cell in the form of secreted and cell surface molecules that are critical in the control of many processes, including the rate of proliferation, and developmental fate of the daughter cells [84]. Early experiments in worms and flies provided the first visualization of the stem cell niche *in vivo*, and subsequent experiments within these systems confirmed the necessity of the niche in regulation of stem cell fate.

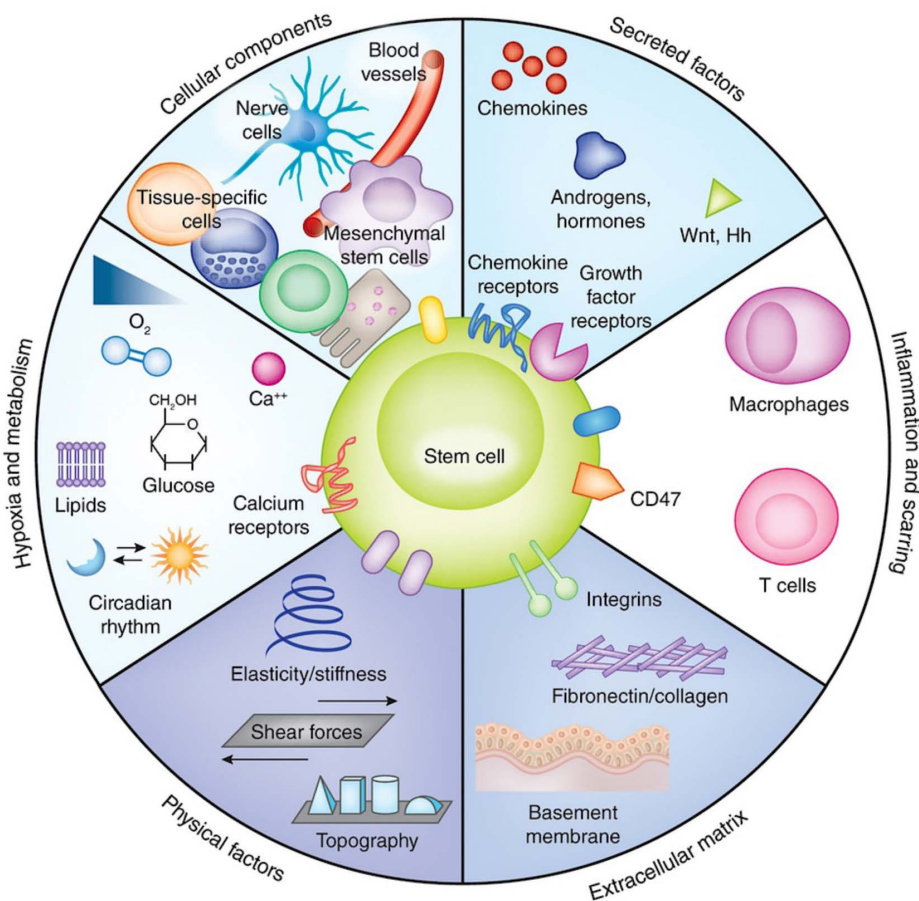


Figure 1.5 Stem Cell Niche Composition

Composition of the niche. Stem cell niches are complex and dynamic structures, which include different, environmentally modulated molecular controls, some of which are secreted factors, physical interaction with ECM, and oxygen concentration.

Figure adapted from Watt et al., 2014.

In mammalian tissues, stem cell niches have started to be characterized and are thought to be just as important as in the early models systems. Each adult stem cell has its own dynamic and independently complex niche [85, 86]. These distinct niches provide support for each stem cell type within the body. The niche can be defined as a subset of tissue, cells, and extracellular substrates that provide support, and can indefinitely house stem cells. In addition to being a home, this niche provides the cues that provide the ability for the stem cell to remain undifferentiated indefinitely (Figure 1.5).

It has also been described that stem cells in normal tissues are capable of self renewal through asymmetrical cell division, while simultaneously generating committed progenitor cells whose descendants may eventually differentiate and carry out tissue specific functions [87]. Of more recent importance, a study of neoplastic tissues has provided evidence of self-renewing, stem-like cells within tumors named cancer stem cells (CSCs). CSCs constitute a small minority of neoplastic cells within a tumor, and are defined by their ability to act as tumor initialing cells and are thought to be supported through discrete niches within tumors [87].

1.4 Role of Extracellular Matrix and Attachment, necessary for engraftment

The biology of the extracellular matrix (ECM) has grown of interest recently due to the increasing body of literature in support of ECM as a means to support stem cell self-renewal through niche mediated biological signaling cascades [48]. These support mechanisms include but are not limited to harboring growth factors, and the support of three-dimensional structure. The field of tissue engineering focuses on creating 3D tissues, which facilitate cell growth, proper organization and subsequent differentiation. Therapeutic applications for 3D biology as a means to regenerative medicine holds much promise, however, arguably there is a long, strenuous road from bench-to-bedside. In order to begin to make these transitions, current signaling biology must be transitioned from 2D to 3D applications, as 3D cell culture provides a necessary feature which allows for mechanical stimulation which is heavily involved in cellular adhesion such as integrin ligation, cell contraction and associated signaling cascades [88]. ECM, integrins, and growth factors as components of cellular niche have many demonstrated roles cell biology, stem cell self-renewal, and matrix specific regulation of cell fate [89].

1.4.1 Integrins as mediators of tissue-specific engraftment

Use of pluripotent-derived cells as therapeutics in regenerative medicine is dependent on their efficiency of integrating within existing tissue, which includes other cells and extracellular matrix components [90, 91]. Tissues and organs have specific architectural and molecular compositions in which cells reside. Engraftable cells will need to rebuild functional tissue architecture in the presence of the existing ECM and cells. Most recent attempts have resulted in disappointing outcomes, as clinical data from a skeletal muscle myoblast study [92] and bone marrow mononuclear cells for myocardial repair [93] have shown that more work needs to be done towards understanding what controls engraftment, and what lineages are necessary [94].

Although some successful observations have been made in engrafting cells in hematopoietic systems [95], this success may not necessarily apply to solid organs. Several other reports have provided exciting proof-of-principle evidence that the seeding of decellularized tissue scaffolds with endothelial and epithelial cells grown in bioreactors can produce bio-artificial lungs [96, 97], livers [98], and hearts [99] that engraft in animals and exhibit normal tissue function for up to several days.

Survival of transplanted cells is of vital importance when considering the use of cells as therapeutics in regenerative medicine, and cellular integrins are the major mediators of cell adhesion and are responsible for activating cell survival by preventing apoptosis [100]. Integrin-mediated cell signaling activates PI3K and AKT, which ultimately regulate cyclin-D to regulate DNA synthesis, among other cellular functions [101]. The repertoire of integrins and ECM binding is cell-type and tissue-type dependent. Functional homing of cells require interactions of a varying range and diversity of ECM ligands with integrin pairs to mediate tissue specific, proper homing. Cells can require multiple attachment integrins as demonstrated by the example of liver sinusoidal endothelial cells which require $\alpha6\beta1$, $\alpha6\beta4$, $\alpha V\beta3$, $\alpha V\beta6$ and $\alpha V\beta5$ and bind multiple ECM ligands [102], or a very finite number of attachment points, while hepatocytes require $\alpha2\beta1$ and $\alpha5\beta1$ binding to collagen and fibronectin [103].

1.5 Extracellular Matrix

In order to understand the cytoskeleton, cell migration, cell growth, cell fate and differentiation it is necessary to understand the structure and the biology of the extracellular matrix (ECM) as well as its composition. A simple definition for the ECM is that it is a structural material that lies under the epithelia and surrounds connective tissue cells [104]. However, it is no longer thought that the ECM is just a support system for the cells that interact with it. Cells have relationships regarding fate to the ECM they create, and the ECM created by the cells surrounding them [105, 106].

The main characteristic that defines ECM regardless of location is the basic structure, which contains a collagen-based scaffold, however the type of collagen, which makes up each tissue specific ECM is different. Adhesive glycoproteins (i.e. laminin and tenascin) adhere to this collagen scaffold and interact with the cells adjacent to the matrix. Interactions between cells and matrix are facilitated by integrins, and ECM is not static, it is constantly undergoing breakdown by matrix metalloproteases [107].

1.5.1 Collagens

Collagens are ubiquitous proteins responsible for maintaining the structural integrity of vertebrates and many other organisms [108]. More than 20 distinct collagens have been identified [109-112]. In tissues that need the ability to resist shear, tensile, or pressure forces, such as tendons, bone, cartilage, and skin, collagen is arranged in fibrils that provide tensile strength. Collagen types I, II, III, V, and XI self-assemble into fibrils [110]. Type IV, VIII and X collagen form networks such as the collagen found in basement membranes [113], and type VI, IX, XII and XIV collagens associate with fibril surfaces collagen [114]. Collagens type XIII and XVIII are more rare, and are transmembrane, and type VI forms a braided structure [110].

Type I collagen occurs throughout the body, except in cartilage. It is the major collagen in the dermis, fasciae, and tendons and is the largest component of mature scar tissue [115].

Type II collagen occurs in cartilage, the developing cornea, and in the vitreous body of the eye [116]. Type III collagen dominates in the wall of blood vessels and hollow intestinal organs, and copolymerizes with type I collagen [117].

1.5.2 Laminin

Laminin, in conjunction with collagen type IV, nidogen, and perlecan, is a major contributing component of basement membrane [118, 119]. The primary role for laminin seems to be mediation of the interaction between cells and the extracellular matrix, notably the basement membrane [118]. Laminin's consist of three disulphide linked chains, and form a characteristic cross shape. To date, five α chains, three β chains and three γ chains have been identified [120]. Of the chain combinations possible, all have not been identified in tissues.

1.5.3 Proteoglycans

Proteoglycans can be grouped into several families which all have a protein core which is surrounded by glycosaminoglycan [121]. The function of proteoglycans was originally assumed to be only structural, as a mechanism to provide protection against compression through mediating rigidity as a structural feature [122], however a member of the proteoglycan family, versican, has roles in addition to just being a structural component and has been shown to stimulate the proliferation of fibroblasts and chondrocytes through its EGF-like motifs [123]. Additionally members of the proteoglycan family are characterized by a protein core which is composed of leucine rich repeats [121, 124] which were first thought to act as facilitators to organization of collagen networks, however it has recently been demonstrated that decorin is involved in signal transduction through the EGF receptor and downstream signaling through the MAPK pathway [125, 126].

1.3.5.1 Heparan sulphate proteoglycans

Heparan sulphate proteoglycans are matrix proteoglycans that are membrane-associated and are predominantly found in basement membranes. FGF ligands are involved with low affinity co-receptors including heparin and heparan sulfate proteoglycans [127-130]. FGF and FGF receptors can bind heparan sulfate proteoglycans, and it is thought that heparan sulfate proteoglycans can act to bind FGF to the receptor to facilitate subsequent signaling [128, 131-134].

1.5.4 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases [135]. There are 23 distinct MMPs expressed in humans, all of which have a general structure of three common domains, and MMPs are subsequently characterized by their architectural features MMP [136, 137]. Fibroblasts express MMP1-3, 9, 11, 13, and 19 [138]. MMPs play a role in various physiological processes including tissue remodeling and organ development [139], in the regulation of inflammatory processes [140] and in diseases such as cancer [141].

1.6 Integrins:

As a family, integrins were first recognized over 25 years ago [142]. They have been identified and restricted to the metazoa, with no homologs present or predicted in plants or fungi [143]. Even within the most primitive metazoan, integrins are present as heterodimers [144, 145].

Integrins have been studied and identified for biological roles as adhesion receptors, mediators of development [144], immune response, and leukocyte trafficking, cancer as well as cancer metastasis [145, 146]. Integrins are a large family of heterodimeric, transmembrane cell surface molecules, which serve as the main receptors for extracellular matrix [100]. Functional integrin receptors consist of an alpha (α) and a beta (β) subunit which dimerize to form

functional heterodimeric receptors [147, 148], interact with ECM, and generate intracellular signal transduction [100]. Integrin receptors are expressed in many cell types and play critical roles in a diverse range of biological processes including cell motility, adhesion, proliferation, apoptosis, development and inflammation (Figure 1.6).

Integrin Receptor Binding

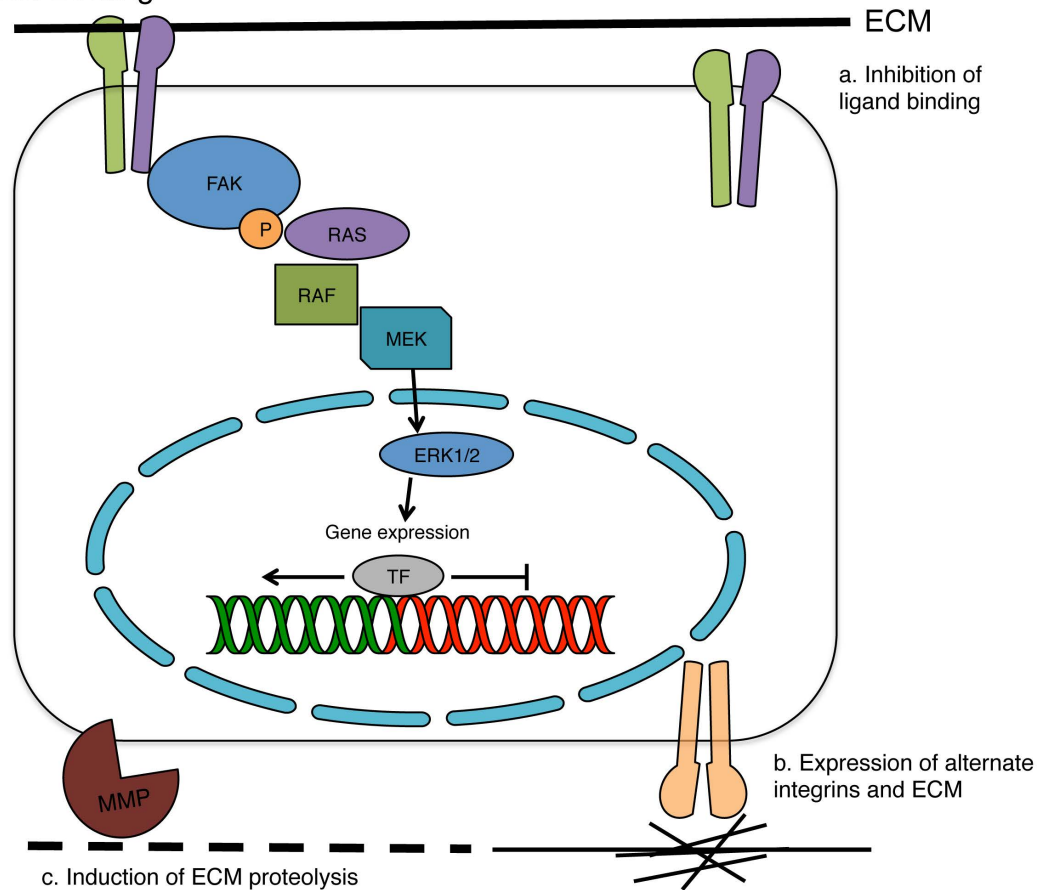
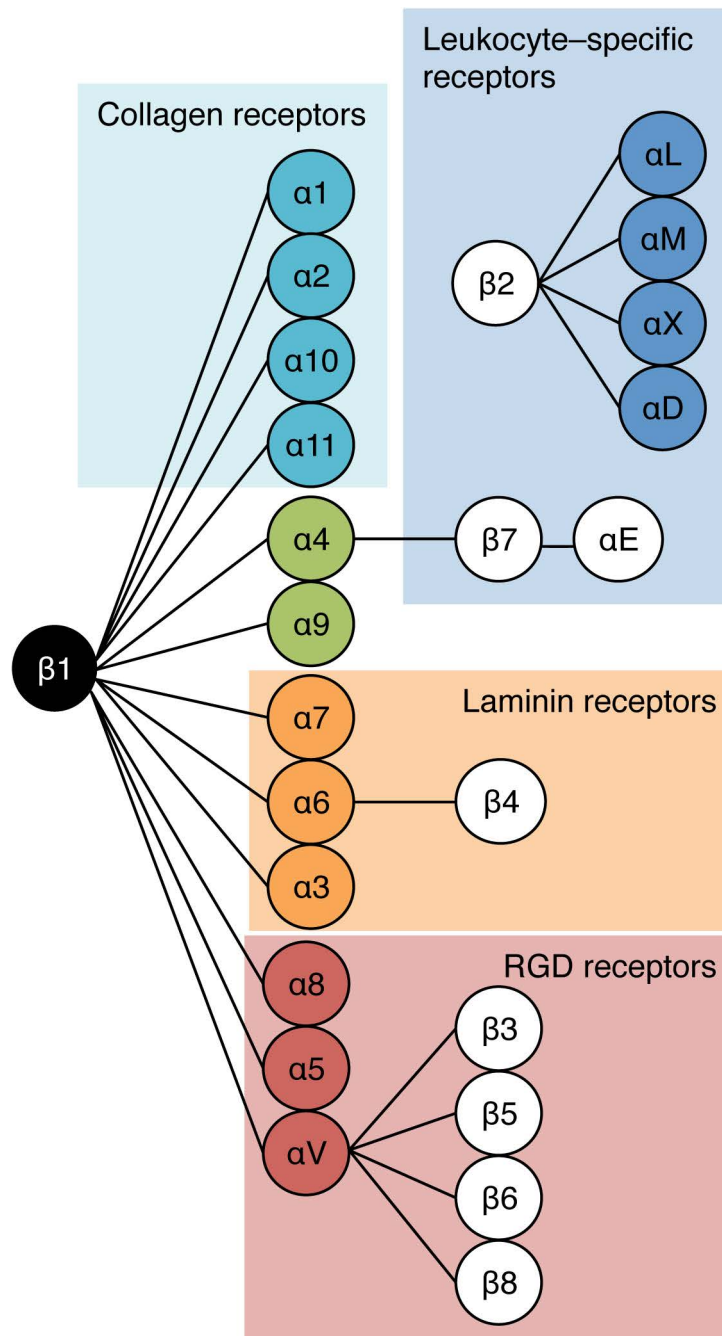


Figure 1.6 Integrins are Bidirectional Signaling Molecules

Upon ligand binding to integrin receptors, focal adhesion kinase pathway can become activated, and this can have several downstream effects through activating or repressive actions of transcription factors. They can (a) cause inhibition of integrin ligand binding, (b) alter the expression of differential integrins and extracellular matrix and (c) induce extracellular matrix proteolysis.



Adapted from Hynes (2002) *Cell* 673-687

Figure 1.7 Integrin alpha and beta pairs and associated receptors

This figure is depicting alpha and beta integrin subunit pairs. Out of the 8 beta subunits, they can pair with 18-alpha subunits to form 24 distinct integrin receptors which each have different extracellular matrix affinities which is depicted here by groupings.

Within vertebrates, 18-alpha (α) and 8-beta (β) integrin subunits have been identified, which can heterodimerize to form 24 distinct $\alpha\beta$ pairs (Figure 1.7). These $\alpha\beta$ pairs connect to either the actin cytoskeleton or to intermediate filaments. Integrin adhesiveness is dynamically regulated and modulated through 'inside out' signaling such that signals that are received outside the cell can be processed intracellularly, and vice-versa (Figure 1.6). Extracellular stimuli, such as chemokines, cytokines, and foreign antigens can activate 'inside out' signaling, and modulate integrin cytoplasmic domains, and subsequent signaling, as well as modulate adhesiveness for extracellular ligands [104, 149, 150].

Both the α and the β subunits of integrins are type I transmembrane glycoproteins with large extracellular domains and single spanning transmembrane domains. Electron microscopy has demonstrated that integrins contain an extracellular globular N-terminal ligand-binding domain, which is necessary for the interaction between the α and β subunits [100, 151]. $\alpha v\beta 3$ was the first to have an X-ray crystal structure identified, and it was shown that the integrin had 'legs' severely bent at the 'genu' or knee, which could create a V-shaped topology, where the head domain was juxtaposed to the membrane proximal region [152, 153]. This discovery was defined as integrin resting, and a large number of integrin subunits have been described to exhibit a conformational change this way as well. This bent conformation has been determined to represent a physiologically low affinity state [152], where activation allowing for rearrangement. Integrins can also change conformational state, resulting in the extension of the head region, and subsequent binding of the extracellular ligand [154, 155] (Figure 1.8).

In addition to different activation states, different integrin pairs have been shown to regulate ECM composition differentially. $\alpha 1$ has been shown to stimulate proliferation in cells [156] and decrease collagen production [157, 158], while $\alpha 2$ has been demonstrated to increase both collagen production and turnover [157-160].

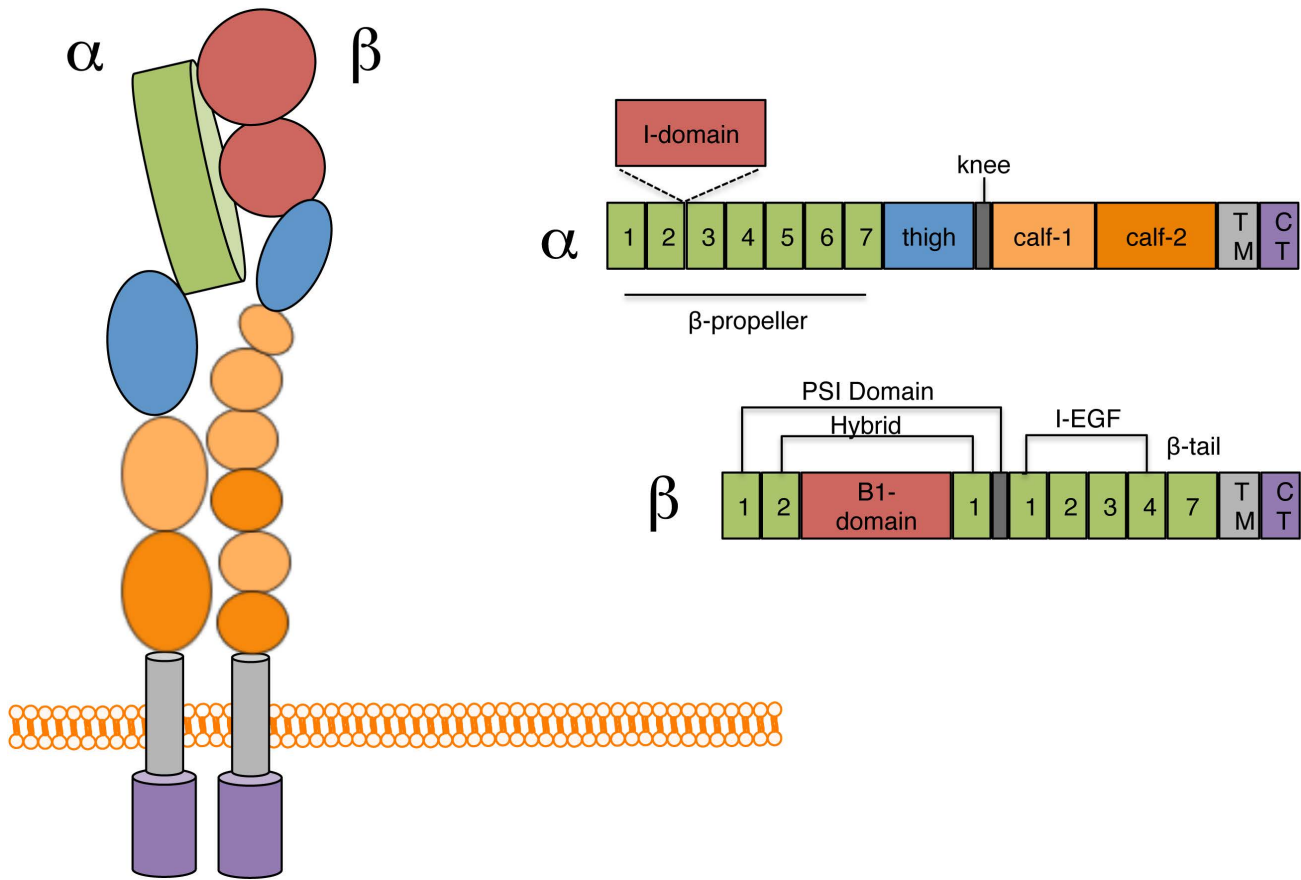


Figure 1.8 Integrin alpha and beta structure

Integrin alpha and beta linear domain structures and integrin dimer structure as an activated dimer. Ligand binding occurs at the Beta-propeller region and Beta-1 domain.

Adhesions mediated by integrins are assembled from a complex family defined by a diverse and elaborate network of structural and molecular regulatory roles. This complex family of regulation is known as the integrin adhesome [161]. This adhesome consists of over 232 components, which can be categorically divided into 148 components which are present directly at the site of adhesion, and another 84 components which can be transiently associated with the adhesion and subsequently its signaling [162]. This large dynamic and complex system is still poorly understood.

1.7 Integrin- alpha 11

Integrin $\alpha 11$ is the most recent member of the integrin family to be discovered. $\alpha 11$ was identified on cultured human fetal myotubes *in vitro* [163]. Additionally a mouse variant has been identified [163]. In muscle, $\alpha 11$ is not present *in vivo*, and expression of $\alpha 11$ has been shown *in vivo* to be through fibroblasts present in native muscle architecture [164]. $\alpha 11$ is an integrin beta-1 pairing integrin, and as a heterodimer $\alpha 11\beta 1$ binds collagen type-I (COL-I) [165]. $\alpha 11\beta 1$ attachment to COL-I has high affinity for COL-I, and low affinity for collagen-IV through co-transfection studies with varying integrin alpha subunits in mouse muscle satellite cells [166-168]. The I domain of $\alpha 11$ recognizes the triple-helical conventional GFOGER motif sequence present in collagen-I [166]. Functionally, $\alpha 11$ has been shown to be involved in cell migration and collagen reorganization [169].

The human $\alpha 11$ gene (ITGA11) is localized on chromosome 15q23 and spans 130kb. Both the human and mouse ITGA11 gene contain 29 introns (Figure 1.9). Human ITGA11 promoter lacks both TATA and CCAAG boxes [167], and the transcription start site is 30 nucleotides upstream of ATG [170]. Promoters of Integrin- α subunits contain conserved initiator elements generally associated with an upstream Sp1 site proximal to the transcription start site [171], which is thought to be necessary for expression due to lack of a TATA box. An Sp1 binding site lies within ITGA11 upstream of the putative initiator sequence. An Sp1 and an Ets-1 site in the proximal promoter of ITGA11 has been shown to be essential for its promoter activity [167] through a screen of promoter deletion constructs which characterized the 3kb region that has been found to have a high level of transcription activity.

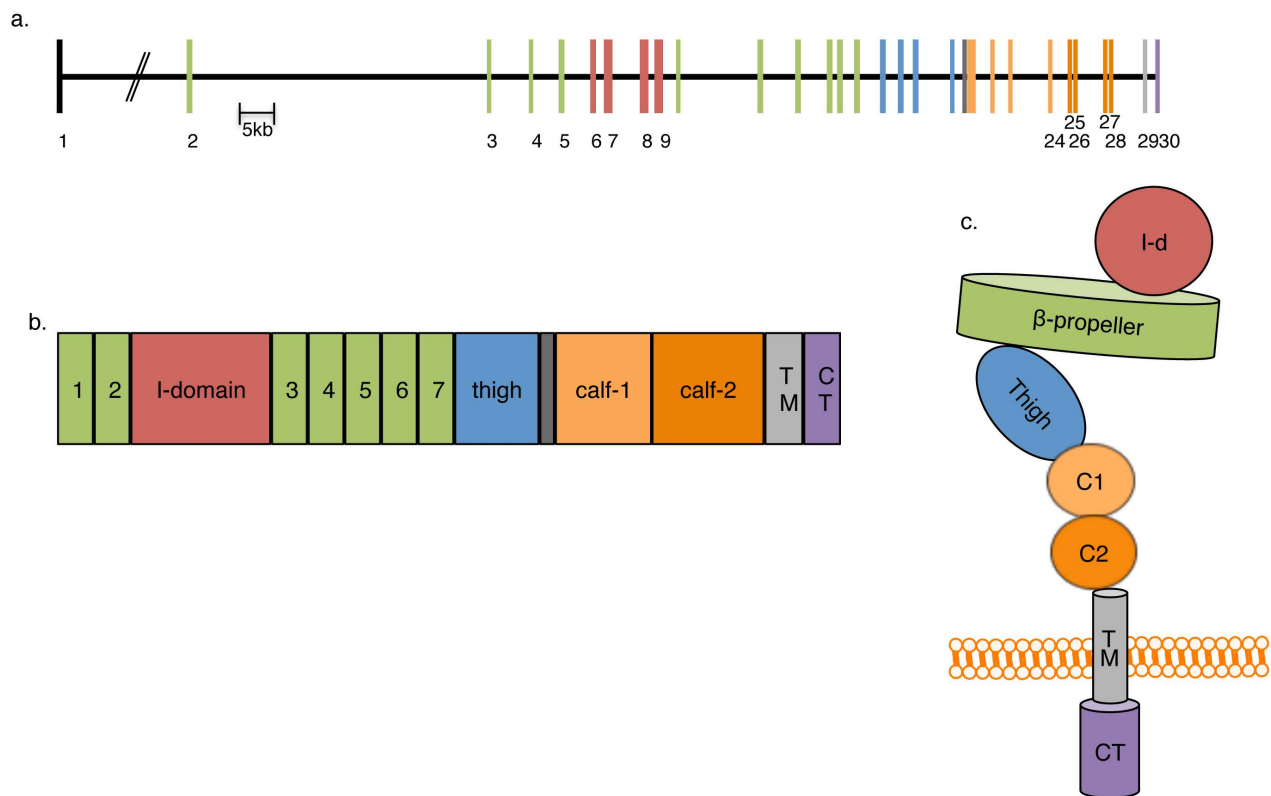


Figure 1.9: Integrin alpha-11 structure

This figure defines the overall structure of integrin alpha 11. (a) schematic overview of *ITGA11* gene which is 130 kb located on chromosome 15q23, and contains 30 exons, (b) schematic overview of α 11 protein, I-domian – I-d, 7-FG-GAP repeats making the β -propeller, calf-1 and calf-2 – C1, C2 respectively, transmembrane domain – TM and cytoplasmic tail – CT. (c) schematic representation of straightened active conformation of α 11 protein with domain structures based on crystal structure of α v integrin, with the I-domain located between the second and third β -propeller repeats.

ITGA11 encodes a mature protein of 1166 amino acids which under reducing, and denaturing conditions runs at 145kD. The extracellular domain contains seven FG-GAP repeats and a 195-amino acid-long I domain inserted between the repeats 2 and 3. The I domain presents a metal ion-dependent adhesion site (MIDAS) motif and three potential divalent cation binding motifs. The cytoplasmic tail contains 24 amino acids, and contains a GFFRS motif, which is different than the GFFKR motif that is commonly found in alpha subunits. Human and mouse α 11 integrin chain shows 89% identity and 97% identity, respectively, within the I domain [172, 173].

In human adult tissue, the $\alpha 11$ transcript is expressed in high levels in uterus and heart and at midrange levels in skeletal muscle [174]. The $\alpha 11$ chain expression is described as being specific to mesenchymal non-muscle cells *in vivo*, but a complete characterization in adult tissues has not yet been performed [175]. As a Col-I binding integrin, $\alpha 11$ shows high levels of expression in more restricted cell types in comparison to other collagen-interacting alpha subunits, as $\alpha 1$ and $\alpha 2$ have been shown to be expressed in a wider array of cells types [176, 177].

1.7.1 Integrin alpha-11 Regulation

Cytokines regulate $\alpha 11$ expression due to TGF- $\beta 1$ responsiveness in Smad2/3 and Sp1-regulated transcription. The ITGA11 proximal promoter contains Smad-binding element SBE2 and the Sp1-binding site SBS1 (Figure 1.10). In transgenic reporter mouse embryos, the human 3kb ITGA11 promoter drives a fibroblast-specific expression [170]. TGF- $\beta 1$ has been demonstrated to up-regulate $\alpha 11$ in a fibrosarcoma cell line (HT1080), in human dermal fibroblasts [178] and fibroblasts (MRC-5) [164, 179]. TGF- β superfamily member Activin-A has also been shown to be a mediator of $\alpha 11$ up-regulation when added to mouse embryonic fibroblasts (MEFs) [170]. IFNs have been shown to stimulate $\alpha 11$ transcription and translation [180, 181]. Type-I interferons, including IFN α and IFN β , have been shown to regulate $\alpha 11$ expression in a human glioblastoma cell line (T98G). Down-regulation of $\alpha 11$ has been reported to occur in mesenchymal stem cells and mouse embryonic fibroblasts treated with FGF2 [182, 183]. However, the responsive elements involved in this down-regulation have not yet been determined in the $\alpha 11$ promoter [175].

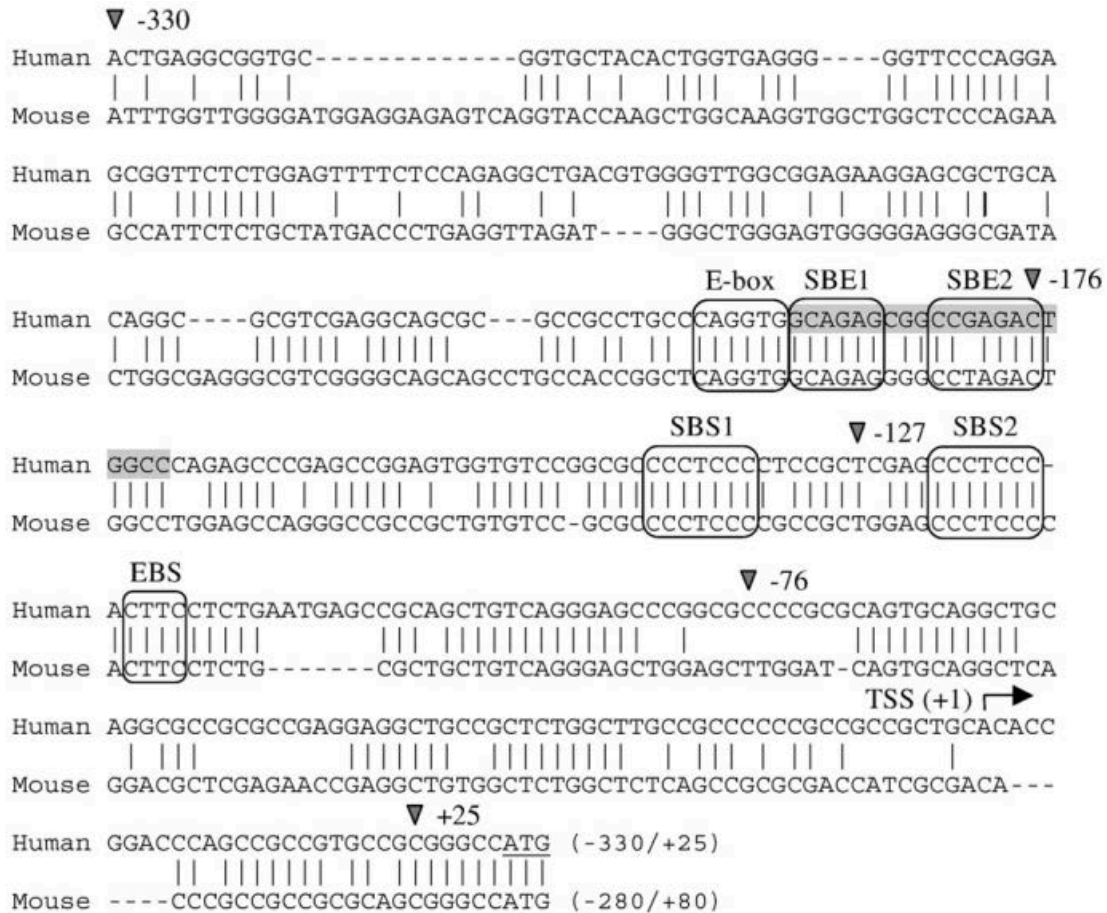


Figure 1.10 Integrin alpha 11 promoter

Figure from Lu et. al., 2010. Integrin alpha-11 human and mouse promoters aligned and mapped. Smad binding elements indicated as SBE1 and SBE2, Ets-binding site (EBS) and transcriptional start site (TSS).

1.7.2 Integrin alpha-11: Function

In vivo functionality of $\alpha 11$ has been shown using a $\alpha 11$ -deficient knockout mouse model. $\alpha 11$ -deficient mice are smaller and have an increased rate of mortality when compared to heterozygous knockouts or wild-type mice [174]. The smaller size of $\alpha 11$ -deficient mice appears to be linked to malnutrition as a result of late incisor eruption and abnormal tooth shape. This hypothesis is further strengthened by the observation that bone formation and development of connective tissue appear normal, and in addition $\alpha 11$ is expressed in *in vivo* at sites where collagens are highly organized [184]. As demonstrated in knockout mouse models, high expression of $\alpha 11$ has been demonstrated in the periodontal ligament [163, 178, 182].

Most recently $\alpha 11$ has also been reported in tumor tissue from melanoma and lung carcinoma [185], and cancer-associated fibroblasts contribute high levels of $\alpha 11$ expression in lung carcinoma. In addition to lung cancer, $\alpha 11$ in a xenograft model $\alpha 11$ was shown to enhance tumorigenicity by up-regulating IGF-2 expression [185]. Yet the role of $\alpha 11$ in cancer-associated-fibroblasts, as well as their role in TGF- $\beta 1$ -dependent myofibroblast differentiation in tumor stroma during subsequent growth and metastasis is unknown.

The detailed molecular mechanism involved in $\alpha 11\beta 1$ function is not well known. This includes the major signaling pathways utilized by $\alpha 11\beta 1$ and its involvement in various pathological conditions and little is known about its role in the MET transition. Further studies of the $\alpha 11$ regulation will be imperative to determine how $\alpha 11$ expression is controlled in fibroblasts, myofibroblasts and cancer-associated-fibroblasts. It is necessary to elucidate the response of ITGA11 to growth factors such as FGF2 and TGF- $\beta 1$ as they are and mediators of fibrotic program, and potential therapeutic targets for adverse fibrotic response such as solid tumor stroma.

1.8 Summary of Thesis

The work contained in the succeeding chapters of this thesis extends the observations published by our laboratory, showing that relatively simple manipulation of cell culture environment *in vitro* can lead to significant changes in the gene expression profile of human dermal fibroblasts (hDF). These cells activate expression of stem cell genes (Oct4, Sox2, Nanog, Rex1 and Lin28), demonstrate increased life span *in vitro*, and acquire regeneration competence (induced regeneration competent cells - iRC). The culture system mediating these changes is characterized by reduced oxygen tension and the addition of exogenous fibroblast growth factor FGF2. While iRC cells exhibit some molecular characteristics of pluripotent cells they do not acquire pluripotency and they do not form tumors when injected into SCID mice. One major characteristic of our *in vitro* culture system is the induction of an extended lifespan. In addition, human muscle derived fibroblasts cultured under the aforementioned conditions contribute to regeneration of mouse skeletal muscle through functional engraftment and acquisition of a PAX7 positive satellite stem cell phenotype. FGF2 appears to be the critical component to this system.

To further characterize how low oxygen-mediated FGF2 affects human fibroblasts, we investigated molecular mechanisms involved in altered expression and production of extracellular matrix and adhesion molecules, and the role of these changes in phenotype switching.

Objective 1

Characterize extracellular matrix and adhesion molecule shifts in human dermal fibroblasts (hDF) undergoing a phenotype shift resulting in induced regeneration competent phenotype (iRC)

In this chapter we test the hypothesis that FGF2 signaling under low oxygen conditions induces changes in the expression of extracellular matrix and adhesion molecules that

reflect the transition in their developmental potential – namely, a departure from producing predominantly a collagen-based environment.

A large number of molecular cues that cells receive *in vivo* are through their extracellular niche, therefore we hypothesize that the role of the extracellular environment on cell behavior *in vitro* is equally critical for induction and maintenance of shifting the cells' developmental potential. We identify an overall deregulation of extracellular matrix (ECM) and adhesion molecules (AM) in human dermal fibroblasts grown in low oxygen with FGF2 supplementation (iRC). An ECM/AM microarray was used as an initial screen, and we identify 15 most significantly deregulated genes and confirmed their deregulation by qRT-PCR. From the 15 targets chosen, we characterized iRC by a change in niche dynamics defined by the most highly deregulated targets belonging to a similar ontology. A 17-fold down-regulation in *Itga11* (collagen-I attachment) and a 5-fold *MMP1* (collagenase type-I) up-regulation led us to hypothesize that iRC cells were remodeling the extracellular niche by removing collagen-I and subsequently, producing and adhering to non-collagen-I based substrate. By Western blot we show a decrease in collagen COL-I from iRC cultures, as well as its binding integrin alpha-11. Additional qRT-PCR analyses for alternate integrin alpha and beta subunits identified expression of integrins that are preferential binding partners for RGD and laminin substrates, which are indicative of a niche with basement membrane components.

Objective 2:

Investigate the mechanism of low oxygen mediated-FGF2 effect on integrin alpha-11 expression

In this chapter we test the hypothesis that the low oxygen-mediated FGF2 signaling is suppressing the transcriptional activity of integrin α 11 either directly or indirectly.

Deposition of collagen-based extracellular matrix by fibroblasts during wound healing leads to scar formation, which is a typical outcome of the healing process in soft tissue wounds.

Increase in collagen production and increase in collagen binding adhesion molecules – integrins – regulated primarily through TGF- β 1 signaling, which in turn is regulated by FGF2. In this chapter we investigate the mechanism of a collagen-I-binding integrin α 11 (ITGA11) down-regulation in response to low oxygen-mediated FGF2 activity. Using RT-PCR, qRT-PCR, Western blotting and immunocytochemistry, we describe significant down-regulation of ITGA11. We show that loss of ITGA11 requires FGF2 induced ERK1/2 activity, and in the presence of FGF2, ITGA11 expression cannot be rescued by TGF- β 1, a potent activator of ITGA11. These results indicate that the ERK1/2 mediated FGF2 down-regulates α 11 by suppressing TGF- β 1 activity. Additionally, we show that FGF2 is capable of overriding TGF- β 1 mediated alpha-smooth muscle actin expression, a marker of pro-fibrotic activated fibroblasts.

Objective 3

Determine if acquisition of induced regeneration competent phenotype in human dermal fibroblasts proceeds through the Mesenchymal to Epithelial Transition (MET), and whether this transition is modulated by integrin alpha-11

In this chapter we hypothesize that the transition between a fibroblast and an induced regeneration competent cell proceeds through a mesenchymal-to-epithelial transition (MET), and that this transition is a consequence of down-regulation of α 11.

In this chapter we characterize the presence of focal adhesions and focal adhesion kinase activity in iRC's, and identify the activation of initiation stage MET genes. These observations indicate that the activation of MET initiation stages may require a shift in extracellular matrix and adhesion molecules. We attempt to confirm a regulatory role for α 11 in this transition, but cannot achieve over-expression of α 11. Similarly, knock-down studies of α 11 through transient transfection remain inconclusive. To facilitate MET, we aggregate and subsequently culture iRC's in 3D aggregates. We show by Western blot and immunostaining that aggregates do not express epithelial cadherin, which is a defining marker for epithelial

phenotype. We argue that while the completion of MET with acquisition of an epithelial phenotype is necessary for induction of pluripotency, understanding the mechanism of a partial transition may lead to development of cell phenotypes for therapy without the associated risk of tumor formation.

Thesis Conclusions

This thesis describes an overall shift in extracellular matrix and adhesion molecules in human dermal fibroblasts undergoing the initiation phase of Mesenchymal to Epithelial Transition during conversion to induced Regeneration Competent cell phenotype. This transition is marked by a loss of $\alpha 11$ and its binding partner Collagen-I by a mechanism that is mediated by ERK1/2 activation and suppression of TGF- $\beta 1$ mediated regulation of $\alpha 11$. Additionally, a shift in the transcript levels of other adhesion molecules demonstrate that iRC are most likely transitioning to attachment to a laminin and fibronectin-based matrix, suggesting that they may be producing a more “pro-regenerative matrix”. We hypothesize that the shift in cells production and interaction with ECM serves as a feedback loop during the phenotype shift, as contact with extracellular matrix and its involvement in the regulation of potency in adult stem cells within the niche has been demonstrated extensively. We also hypothesize that this “pro-regenerative” shift can be exploited in wound healing biology, as it triggers a non-fibrotic response in dermal fibroblasts, overriding TGF- $\beta 1$ -mediated fibrotic response. As both, TGF- $\beta 1$ and FGF2 are present at the wound site, manipulation of the dynamic relationship between the two could be used to reduce scar deposition.

In summary, our cell culture system allows for a cell-fate conversion from an abundant adult differentiated cell source to a more ‘developmental’ plastic cell type. This cell phenotype has a potential for therapeutic applications in regenerative medicine. Identification of molecular mechanisms controlling this phenotype conversion will allow development of strategies for *in situ* manipulation of wound healing outcomes.

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Chapter 2

Characterization of Extracellular Matrix and Adhesion Molecules in Human Dermal Fibroblasts Undergoing Induced Regeneration Competence

2.0 Abstract

A large number of molecular cues that cells receive *in vivo* are through their extracellular niche, therefore **we hypothesize that the role of the extracellular environment on cell behavior *in vitro* is equally critical for induction and maintenance of cells which are shifting their developmental potential.** In this chapter we identify an overall deregulation of the extracellular matrix (ECM) and adhesion molecules (AM) that accompanies an extended lifespan phenotype in human dermal fibroblasts grown in low oxygen with FGF2 supplementation, that we have named induced regeneration competence (iRC). An ECM/AM microarray was used as an initial screen that identified the 15 most significantly deregulated genes, and confirmed their deregulation by qRT-PCR. We characterized iRC by a change in niche dynamics defined by the most highly deregulated targets belonging to a similar ontology. A 17-fold down-regulation in *Itga11* (collagen-I attachment) and a 5-fold *MMP1* (collagenase type-I) up-regulation led us to hypothesize that iRC cells were remodeling the extracellular niche by degrading collagen-I and subsequently producing and adhering to non-collagen-I based substrate. Western blot analysis showed a decrease in collagen-I (COL1) protein in iRC cultures, as well as a decrease in its binding integrin alpha-11. Additional qRT-PCR analyses for alternate integrin alpha and beta subunits identified up-regulation in expression of integrins that are preferential binding partners for RGD and laminin substrates, which were indicative of a niche comprised of basement membrane components.

Objectives and Hypothesis

The objectives of this aim were to 1) characterize the changes in expression of extracellular matrix and adhesion molecules in human dermal fibroblasts (hDF) undergoing a phenotype shift resulting in induced regeneration competent phenotype fibroblasts (iRC), and 2) test the hypothesis that FGF2 signaling under low oxygen conditions induces changes in the expression of extracellular matrix and adhesion molecules that reflect the transition in their

developmental potential – namely, a departure from producing predominantly collagen-based environment.

2.1 Introduction

Regenerative medicine has been receiving an increasing amount of attention over recent years due to the clinical and commercial potential. The successful derivation of human embryonic stem cells (hESC) was groundbreaking [1], however hESC have limitations in their potential for therapeutic application as they are a non-autologous cell source and they have raised ethical debates in relation to their source and subsequent derivation [2]. The development of induced pluripotent stem cells (iPSC) [3, 4] created the ability to derive a pluripotent cell from a somatic cell source, which in turn increased the momentum of regenerative medicine research. Research focus was able to shift to cell based applications because the potential range of applications for an autologous, pluripotent, cell source is far broader for cell therapy and disease modeling than hESC.

The discovery of iPSC [5] led to a sustained effort from many research groups to try to develop novel derivation strategies. However, major hurdles still exist before iPSC-derived cell therapy can be used in patients. DNA-integrating viruses were first used to create iPSC, but this introduces genomic alterations deeming these cells non-clinically compliant [6]. Recent protocols achieve the reprogramming using non-integrative methods such as Sendai virus [7], non-integrating adeno-associated virus [8], episomal vectors [9], microRNAs, protein delivery [10] or PiggyBac transposons [11, 12]. This progress has eliminated the potential for virally induced tumor formation, and these methods are able to minimize genomic perturbations [13] however, iPSC derived with the aforementioned protocols all come with individual limitations [14]. A common limitation is that they all have low reprogramming efficiency.

Interest and efforts so far have been focused on using exogenously added factors to induce cellular reprogramming, however the interest in methods for derivation and maintenance of stem cells has shifted its focus to the importance of environmental cues, with emphasis on the role of extracellular matrix (ECM) and adhesion molecules (AM) [15]. However in order to

use the potential of the environment to direct the cell-fate, it is necessary to understand the role of the extracellular environment on stem cell pluripotency, maintenance of pluripotency, and behavior. It is well documented that one of the most critical proteins involved in the derivation, and maintenance of hES and iPSC is basic fibroblast growth factor (FGF2) [16-18], and its bioavailability and activity is mediated by the extracellular matrix proteoglycan, heparan sulfate [19-23].

During our work in cellular reprogramming, we have shown that relatively simple manipulation of the cell culture environment *in vitro* can lead to many changes in the gene expression profile of these cells [24]. Moreover, these changes result in an iRC phenotype which we define by the induction of expression of stem cell genes (Oct4, Sox2, Nanog, Rex1 and Lin28) and an increase the life span of human dermal fibroblasts [25]. This culture system is characterized by reduced oxygen tension and the addition of exogenous FGF2. Human dermal fibroblasts (hDF) cultured under those conditions exhibit some molecular characteristics of pluripotent cells; however they do not acquire pluripotency and do not form tumors when injected into SCID mice [25]. One major characteristic of our *in vitro* culture system is the induction of an extended lifespan. In addition, human muscle derived fibroblasts cultured under the aforementioned conditions contribute to regeneration of mouse skeletal muscle through functional engraftment and acquisition of a PAX7 positive satellite cell phenotype [26]. As with maintenance of pluripotency, FGF2 appears to be the critical component to our system.

In humans, five FGF2 isoforms can be produced (18, 22, 22.5, 24 and 34 kDa), which display different subcellular localization and contribute to different cellular behaviors [27, 28]. The 18 kDa FGF2 isoform signals through ligand-dependent activation of its receptor tyrosine kinase family [29], and is the isoform used throughout our studies.

Based on our previous data, we hypothesized that changes in the ECM and AM expression profile may play a role in the phenotypic shift of an hDF to an iRC, resulting in a niche that is permissive to a more plastic state. A large amount of the molecular cues that the

cells receive *in vivo* are through their extracellular niche [30], and the role of the extracellular niche on stem cell behavior *in vitro* is equally as critical for maintenance of pluripotency. Stem cells (adult and embryonic) reside in dynamic yet specialized microenvironments, which provide molecular cues that control the balance between self-renewal and differentiation [31-33]. Differential expression of extracellular matrix proteins serves to mediate the specificity of the stem cell to its niche [32, 34]. Extracellular matrix proteins are also involved in mediating homing to the niche and serve as a reservoir for growth factors and cytokines [35]. Molecular cues that are involved in maintenance of plasticity come from cell-cell contact, as well as cell-matrix contact [33, 36, 37]. Interplay between integrins and the niche are known to support tissue specific stem cells through the interaction of cadherins and integrins specifically [38].

To support pluripotency of hESC and iPSC *in vitro*, it is known that in lieu of a feeder layer, murine basement membrane can support pluripotency. Classically, stem cell fate was thought to be controlled by genetic and molecular mechanisms, however we now know that the extracellular environment is arguably equally important in stem cell fate determination through ECM and AM. Integrins are responsible for transmitting extracellular cues through intracellular signaling [39].

A major contributor to the signaling from ECM / AM are integrins. Integrins are a large family of heterodimeric, transmembrane cell surface molecules which serve as the main receptors for ECM [39]. Functional integrin receptors consist of one alpha (α) and one beta (β) subunits, which form biologically functional receptors [40, 41] that can interact with the ECM, and can result in the activation of intracellular signal transduction pathways [39]. Integrin receptors are expressed on many cell types and play critical roles in a diverse range of biological processes including cell migration, adhesion, proliferation, apoptosis, development, and inflammation [42]. Integrins are more than just cell adhesion molecules; they transmit bi-directional signals across the plasma membrane [39]. Integrin adhesiveness is dynamically regulated and modulated through 'inside out' signaling such that signals that are received

outside the cell can be processed intracellularly, and vice-versa. Extracellular stimuli such as, chemokines, cytokines, and foreign antigens can activate 'inside out' signaling, and modulate integrin cytoplasmic domains, and subsequent signaling, as well as modulate adhesiveness for extracellular ligands [43-45]. Due to the dynamic range of bi-directional signals that integrins can transmit, and the complexity of the system, much is yet to be discovered about what integrin signaling mediates.

This section of my thesis defines the overall changes in the composition ECM and in the integrin profile of human dermal fibroblasts, which is ultimately characterized by the loss of collagen I protein as well as the loss of integrin alpha-11 expression.

2.2 Results and Discussion

ECM/AM microarray

A microarray for ECM/AM targets (SA Biosciences) was used to compare the expression of ECM/AM cDNA levels between human embryonic stem cells (hESC), and human dermal fibroblasts grown with (iRC) or without FGF2 (hDF) (Figure 2.1). A total of 96 target genes were analyzed, and 25 of them were determined to be detectable in treatments and controls. Fifteen most highly changed genes were chosen for subsequent validation by qRT-PCR (Figure 2.3a). When normalized to hESC, iRC showed a trend towards the gene expression profile of hESC, which was demonstrated with a 17-fold down-regulation of integrin alpha-11 ($\alpha 11$) and a 5-fold up-regulation in matrix metalloproteinase-1 (MMP1) (Figure 2.2). This suggested that iRC might be building a less collagen-I rich matrix mediated by MMP1 activity.

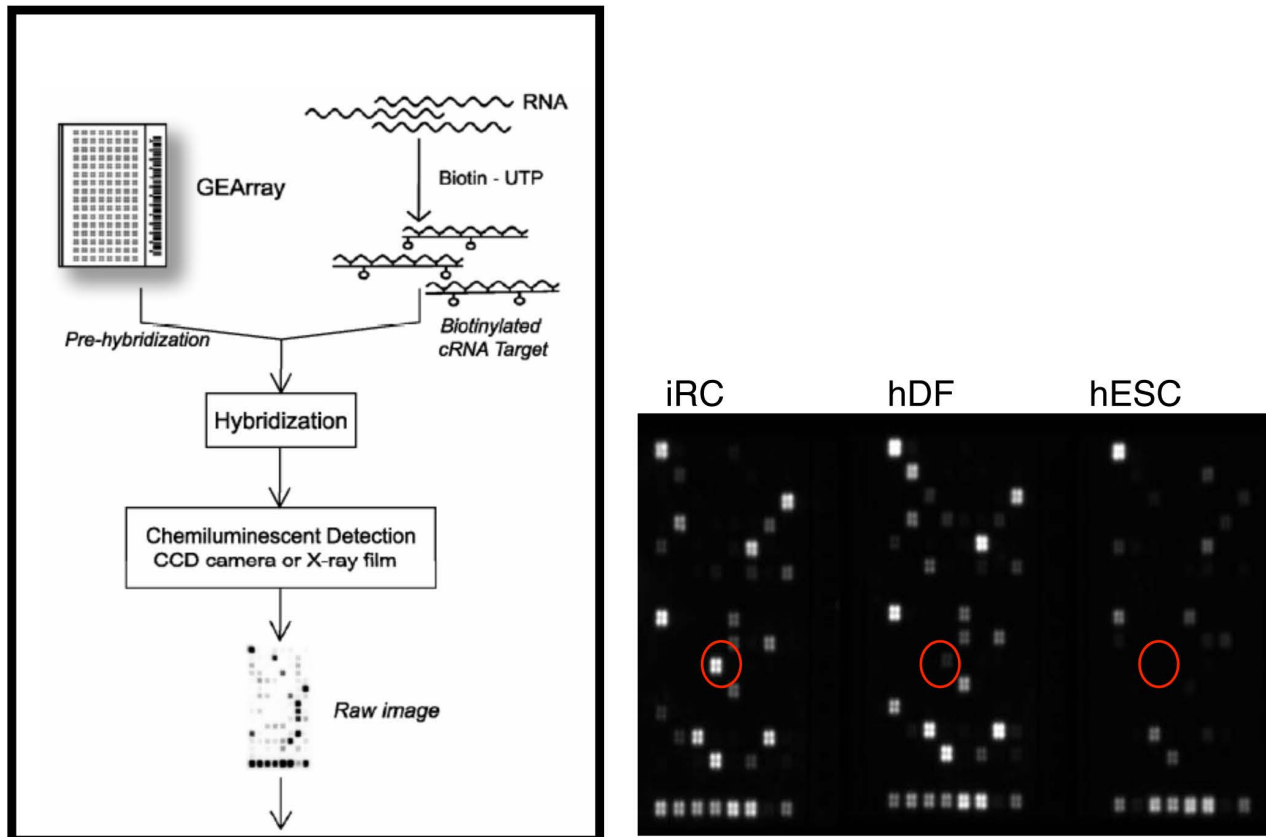


Figure 2.1 Schematic Extracellular Matrix and Adhesion Molecule Array

A human extracellular matrix and adhesion molecule hybridization array containing 96 target genes.

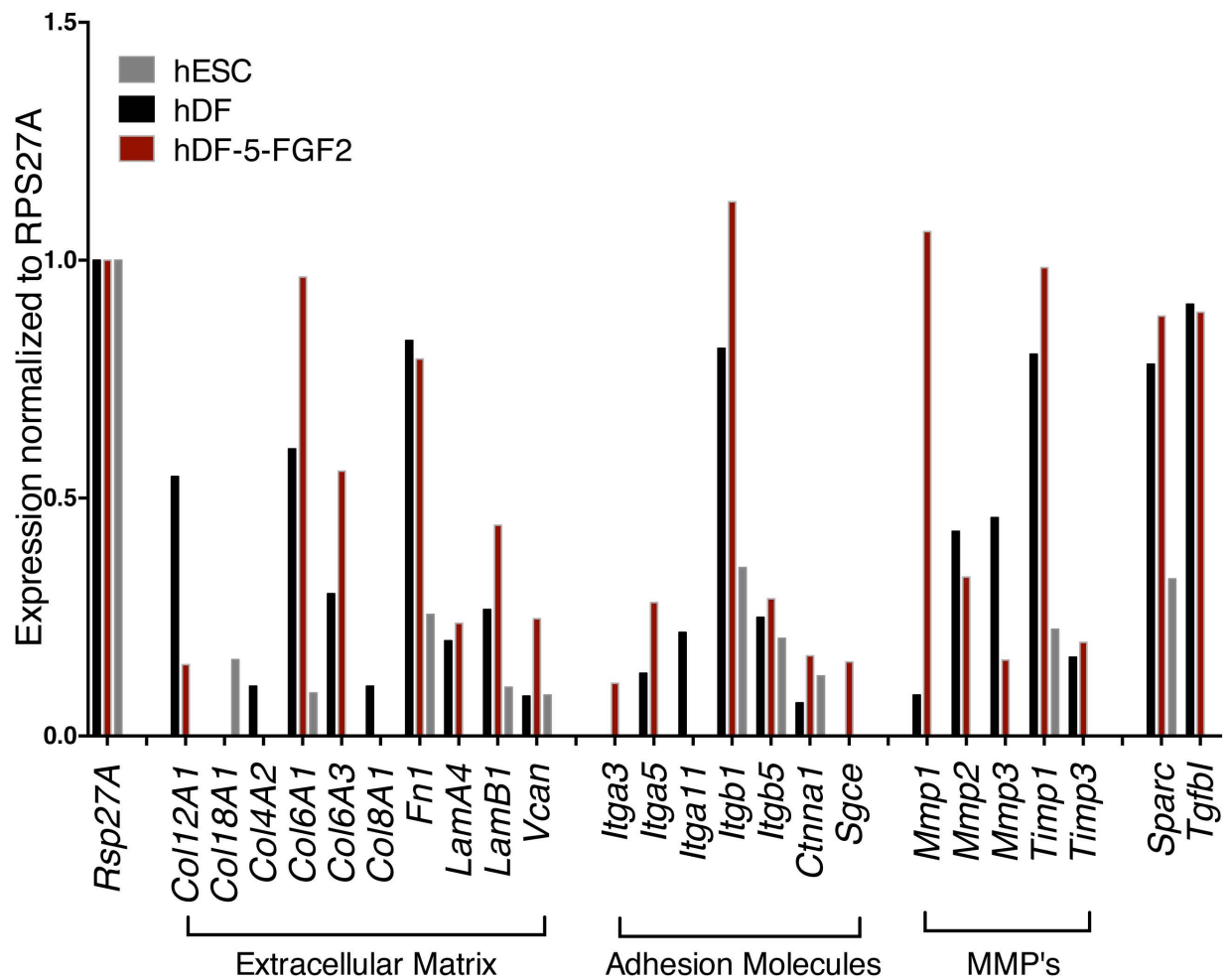


Figure 2.2 Effect of FGF2 on expression of extracellular matrix and adhesion molecules

Expression was assayed in fibroblasts grown for 30 days in 5% oxygen without FGF2 (hDF-5) or with FGF2 supplementation (hDF-5-FGF2) and human embryonic stem cells (hESC) by Extracellular Matrix and Adhesion Molecule Array. Data were normalized to ribosomal protein Rsp27A using densitometry.

qRT-PCR confirmation of array targets

qRT-PCR was used to validate the results of the ECM/AM array for the 15 most highly deregulated targets in hDF, iRC, and hESC. FGF2 supplementation under 5% oxygen resulted in a 17-fold down-regulation in *Itga11*, and a 35-fold up-regulation in interstitial collagenase matrix metalloproteinase-1 (*Mmp1*) (Figure 2.3a), which is responsible for collagen-1 degradation. Additionally, we observed a 2-fold up-regulation in *Itgb1* and a 4-fold down-regulation in *Fibronectin 1 (Fn1)* (Figure 2.3a). Collectively, this data suggests that in human dermal fibroblasts, treatment with FGF2, under low oxygen conditions induces a shift away from a collagen I based attachment. Deregulation of expression for the majority of the ECM and adhesion molecules profiled by qRT-PCR was induced by lowering the oxygen concentration to 5%, and this effect was further potentiated by the addition of FGF2 (Figure 2.3a).

The observation that iRC attachment protein expressions were not significantly down-regulated prompted us to analyze expression of other α/β integrins in order to determine if other integrin pairs were substituting for *Itga11* activity. Comparison of hDF cultures with iRC showed an increase in the transcript level for several integrin subunits ($\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 1$, $\beta 3$, $\beta 5$) (Figure 2.4) with up-regulation of $\alpha 6$ and $\beta 3$ with the being most highly up-regulated by 10- and 4-fold respectively.

Out of the up-regulated integrin subunits identified, the potential integrin pairs were identified as follows: $\alpha 6\beta 1$ and $\alpha 6\beta 4$ are receptors for laminins, which are predominately present in basement membrane [46], and $\alpha V\beta 3$ and $\alpha 5\beta 1$ are RGD integrin receptors, and are receptors for fibronectin [47] and vitronectin [48] respectively. In summary, when comparing potential pairs for all up-regulated α and β subunits, substrates being bound are likely laminins, and RGD receptor-binding pairs, fibronectin, vitronectin.

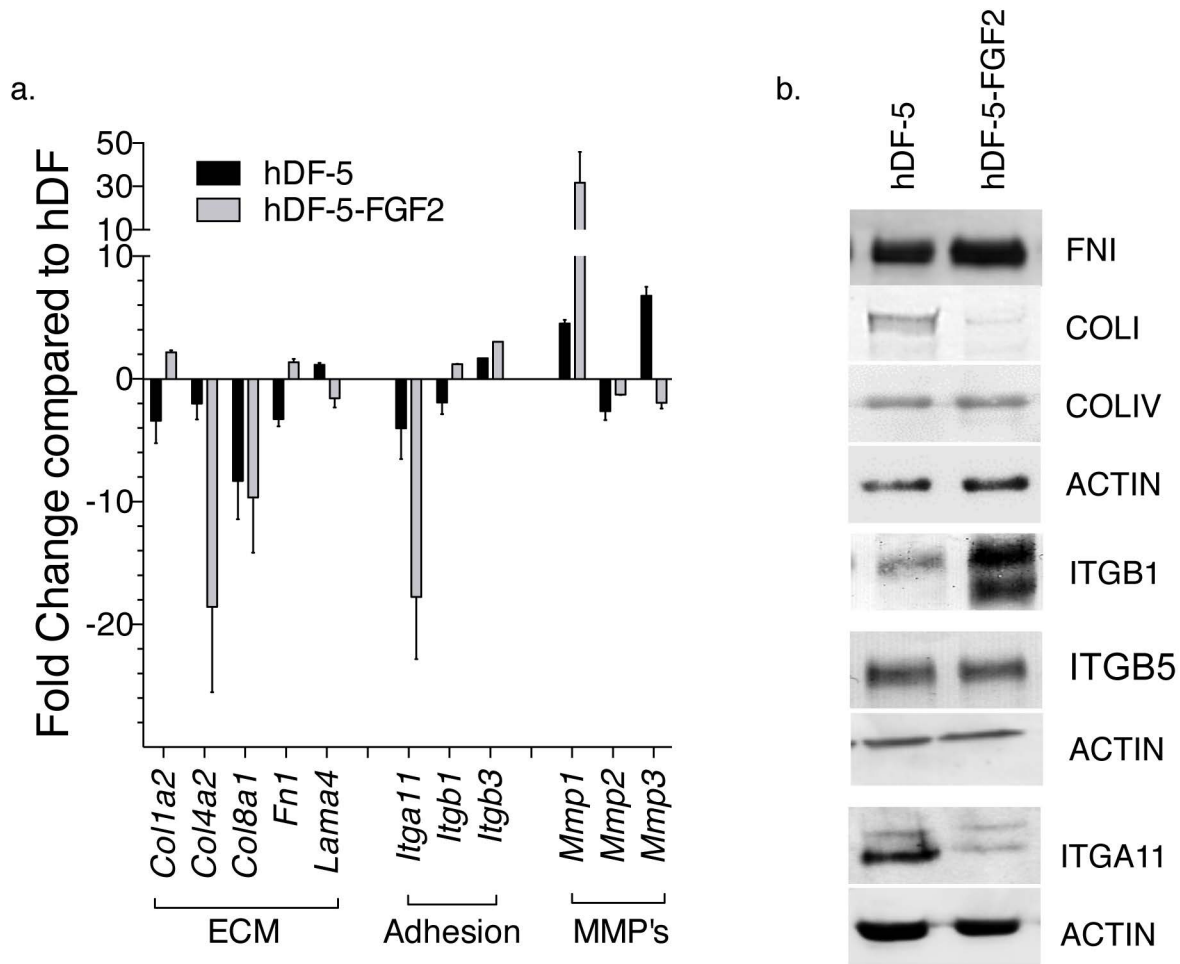


Figure 2.3. Effect of FGF2 on RNA expression levels of extracellular matrix and adhesion molecules.

(a) Expression was assayed in fibroblasts grown for 30 days in 5% oxygen without FGF2 (hDF-5) or with FGF2 supplementation (hDF-5-FGF2) by qRT-PCR. Data were normalized to actin in control fibroblasts grown in atmospheric oxygen without FGF2. Expression levels are presented as fold change in expression using delta-delta CT method. (b) Western blotting for select ECM and AM proteins. Data were normalized to actin in fibroblasts grown in atmospheric oxygen without FGF2 (hDF). FN1 – fibronectin; COLI - collagen I, COLIV – collagen IV, ITGA11 - integrin α 11, ITGB1 – integrin β 1. Error bars indicate SD (n=3)

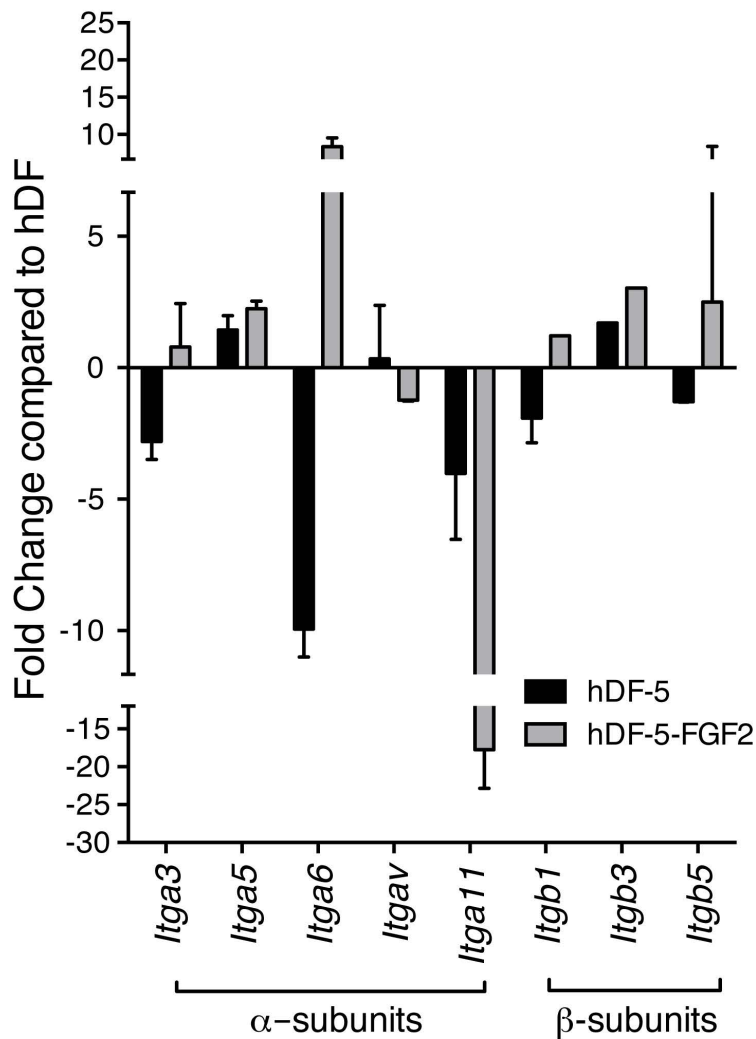


Figure 2.4. Expression of integrin alpha and beta subunit RNAs by qRT-PCR

Levels of integrin alpha and beta subunit RNAs were assayed in fibroblasts grown for 30 days in 5% oxygen without FGF2 (hDF) or with FGF2 supplementation (hDF-5-FGF2) by qRT-PCR. Data were normalized to actin in control fibroblasts grown in atmospheric oxygen without FGF2. Expression levels are presented as fold change in expression using delta-delta CT method. Error bars indicate SD (n=3).

Western blot: ECM/AM targets

Signal transduction pathways triggered by differential binding of ECM have effects on cell behavior, such as proliferation, survival/apoptosis, shape, polarity, motility, gene expression and differentiation [49]. To determine if the transcriptional changes induced by FGF2 and low oxygen resulted in changes in protein levels, we characterized levels of protein expression by Western blotting (Figure 2.3b). Correlating gene expression levels with protein expression

indicated that for a few of the targets, more specifically FN1 and ITGA11, the regulation happens at the transcriptional level (Figure 2.4b).

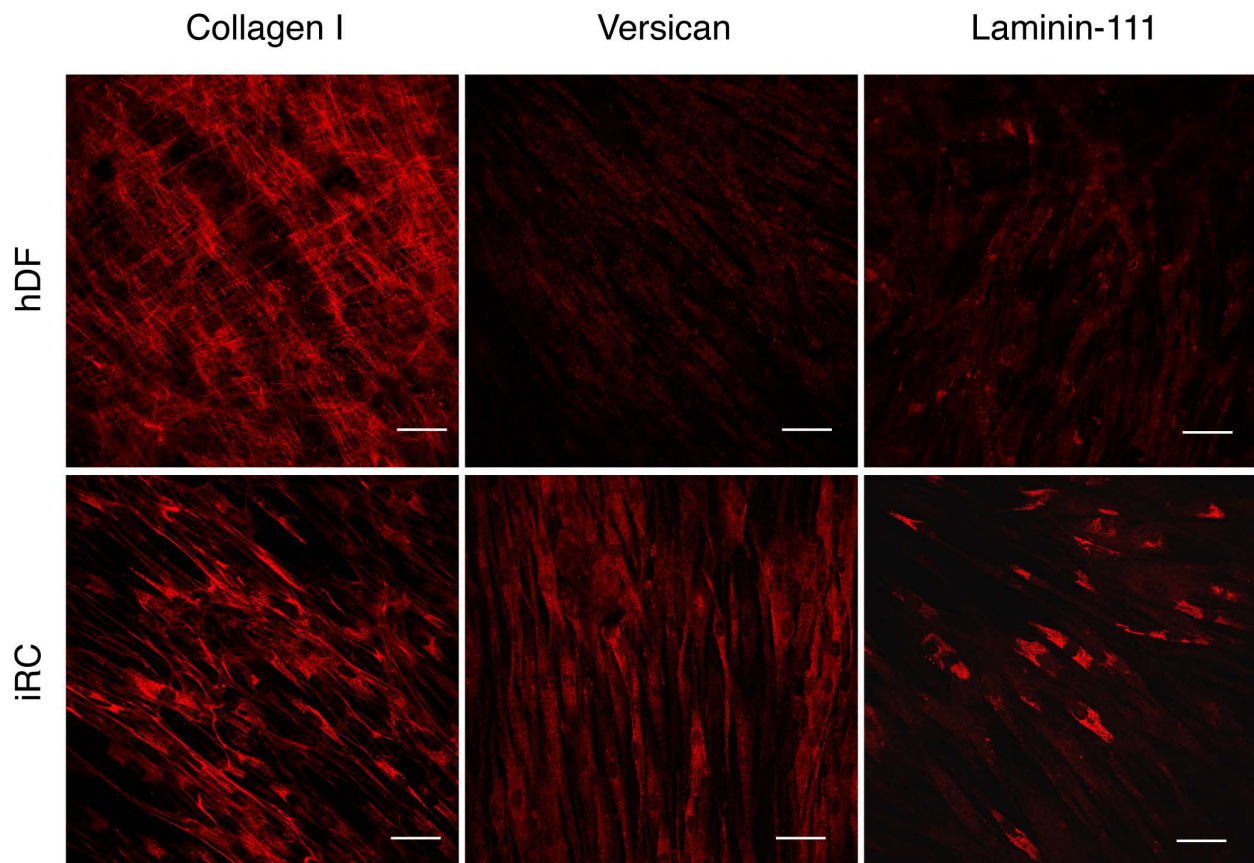


Figure 2.5. Immunocytochemistry of ECM deposited by hDF and iRC

Immunocytochemistry of fibroblasts labeled with primary antibodies against collagen-I, laminin-111 and vitronectin and detected with secondary antibodies conjugated to AlexaFluor568 (red). Images taken at 10x magnification, scale bar indicates 100 μ M.

When grown in the presence of FGF2, fibroblasts decreased production of COL1 and increased production of ITGB1 significantly, while levels of COL4 remained unchanged (Figure 2.3b). However, protein expression levels for COL1, COL4, and ITGB1 levels did not correlate with transcript levels (Figure 2.3b).

Considering the major effects that addition of FGF2 had on altering gene and protein expression levels, we decided to make FGF2 the main variable for our studies. To further

investigate the role of FGF2 on modifying the niche of primary human dermal fibroblasts, we assayed expression of several other proteins hypothesized to be involved in regulating the cellular niche, and saw a decrease in protein levels of ITGA11, COL1, and an increase in FN1, FAK, TGF- β 1 and TGF- β 2. Levels of COLIV remained consistent across all treatments (Figure 2.3b).

While the observed decrease in collagen-I protein expression does not correlate to a decrease in message level, the increase in MMP1 expression suggested that COL1 maybe regulated by MMP1 proteolytic degradation. Western blot analysis for α 11, and COL1 confirmed that they were both down-regulated in cells treated with FGF2 under low oxygen conditions only, and no change was observed when no FGF2 was present (Figure 2.3b). This observation confirmed that the change in α 11, and COL-I expression was an FGF2-mediated effect. Additionally, we looked at the overall localization of COL-I, Versican and Laminin-111 in hDF and iRC cells. In iRC cells, Col-I appeared to have a change in organization, while Versican and Laminin-111 seemed to be more highly expressed when in low oxygen with FGF2 (iRC) (Figure 2.5). This further indicates that low oxygen and exogenous FGF2 experimental culture conditions are creating a functional shift in the ECM/AM of hDF.

2.3 Conclusions

Here we show that with the addition of exogenous FGF2, hDF alter the transcription and translation of extracellular matrix proteins, adhesion molecules and matrix remodeling enzymes.

Attachment to ECM is of importance in many biological situations, of which, human embryonic stem cells are a model for maintenance of pluripotency *in vitro*. hESC colonies are comprised of highly adherent pluripotent progenitors. Dissociation of cell-cell attachments leads to an increased rate of differentiation, and cell death [50]. This suggests that the extracellular niche, as well as cell-cell junctions play a major role in the assembly and subsequent maintenance of pluripotent human embryonic stem cell colonies. In addition to attachment, human embryonic stem cells are dependent on the addition of basic fibroblast growth factor 2 (FGF2), from either exogenous supplementation, or mouse embryonic fibroblast conditioned culture medium [51].

Just as attachment of hESC is known to play a role in the maintenance of pluripotency, a specialized niche is important for phenotype maintenance of adult stem cells [37]. Adult stem cells are responsible for the growth, maintenance and overall homeostasis of most adult tissue types within the body [52-55]. The survival and maintenance of multipotency is regulated by the microenvironment in which they reside [56-59]. Each adult stem cell has its own, dynamic and independently complex niche [60, 61]. The niche can be defined as a subset of tissue, cells, and extracellular substrates that provide support, and can indefinitely house stem cells. In addition to being a place of local homing, this niche provides the cues that provide the ability for the stem cell to remain undifferentiated indefinitely [62].

When analyzing the overall changes in the expression of ECM proteins based on the ECM/AM array profile, we saw a down regulation in *Col12A1*, *Col4A2*, and *Col8A1* in iRC's. Conversely, we saw an up-regulation in *Col6a1* and *Col6A3*. qRT-PCR analysis comparing gene expression level between hDF and iRC, showed an increase in the transcript level for

several integrin subunits when FGF2 was present in the culture medium. More specifically integrin $\alpha 6$ and $\beta 3$ were the most highly up-regulated by 10- and 4-fold, respectively. Down-regulation in integrin receptor transcript levels as well as changes in the expression levels of matrix remodeling proteins such as MMP's (up-regulated) argue that iRC cells are undergoing a remodeling of extracellular matrix attachment. We observed an up-regulation in *Sparc* which has been implicated in both development and injury response with biological implications in cell shape, growth factor efficacy, and ability to affect the expression of MMPs [46]. From the transcriptional changes observed we hypothesize that iRC cells are shifting their attachment away from collagen-I and likely to laminins.

The novel fibroblast phenotype observed here can have several important biological implications especially considering that fibroblasts represent the most abundant cell type among the various cell types that participate in the wound healing process [63]. After injury, fibroblasts replace the wound with granulation tissue, composed of fibronectin and collagen-I [64]. As the wound is re-vascularized, fibroblasts acquire an activated, highly contractile phenotype to close the wound with deposition of collagen-I dense scar tissue [63]. Subsequent to wound closure, remodeling events occur, which induce a change of collagen-I dense scar tissue into more functional tissue architecture. In extreme cases when routine healing process is compromised due to severe trauma, or upon deregulation normal fibrotic/healing response; irreversible fibrosis occurs, resulting in deposition of excessive fibrous connective tissue and dense scar formation. Currently, choric fibrosis is irreversible [65].

The fibroblast phenotype described here, which no longer synthesizes nor anchors to collagen-I, is in line with a fibroblast phenotype that is able to participate in wound healing with a less fibrotic response. Previous work from our lab using the same culture conditions has demonstrated reduced collagen deposition in an *in vivo* mouse skeletal muscle regeneration model [26]. That data serves as a characterization to support the improved functional outcome, which when combined with the molecular characterization described in this part of the thesis

allows us to hypothesize that iRC are becoming less likely to participate in scar formation, and the iRC fibroblast may be able to participate in wound healing with a less fibrotic response. Furthering our understanding of the molecular mechanisms which are controlling the ECM / AM shift we see when fibroblasts are transitioning from hDF to iRC may provide useful insight into potential therapeutic targets for fibrotic conditions such as scarring.

2.4 References

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Chapter 3

Analysis of an Erk1/2 Mediated Down-Regulation of Integrin Alpha-11 in Induced Regeneration Competent Cells

3.0 Abstract

Deposition of collagen-based extracellular matrix by fibroblasts during wound healing leads to scar formation - a typical outcome of the healing process in soft tissue wounds. The process can, however, be skewed in favor of tissue regeneration by manipulation of the wound environment. Under low oxygen and FGF2 supplementation, fibroblasts dramatically alter expression of many genes among which the most significantly deregulated are extracellular matrix and adhesion molecules. **Therefore we hypothesize that low oxygen conditions and supplementation with FGF2 can provide extracellular cues that drive wound fibroblasts towards a pro-regenerative phenotype.** Here we investigate the mechanism of a collagen I binding integrin $\alpha 11$ (ITGA11) decrease in response to low oxygen-mediated FGF2 effects in dermal fibroblasts. Using RT-PCR, qRT-PCR, Western blotting and immunocytochemistry, we describe significant down-regulation of ITGA11. We show that loss of ITGA11 requires FGF2-induced ERK1/2 activity, and in the presence of FGF2, ITGA11 expression cannot be rescued by TGF β 1, a potent activator of ITGA11. Our results indicate that FGF2 may be redirecting fibroblasts towards an anti-fibrotic phenotype by overriding TGF β 1-mediated ITGA11 expression.

Objectives and Hypothesis

The objective of this work is to investigate the mechanism of the low oxygen mediated-FGF2 effect on integrin alpha-11, with the hypothesis that FGF2 signaling is suppressing transcriptional activity of integrin alpha-11 either directly, or indirectly.

3.1 Introduction

Regulation of cell fate through modulation of ECM leading to altered mechano-biological cues suggests that extracellular matrix and binding with integrin is involved in cellular phenotype [1]. Recent discoveries in stem cell biology describe how the modulation of extracellular matrix and the changes in mechano-biological cues from the structural properties of extracellular matrix composition affect stem cell fate. While stem cell fate (differentiation or maintenance of multipotent and pluripotent cell phenotypes) *in vitro* can be controlled by genetic and molecular mechanisms, involving the presence of growth factors for pluripotency support, and transcription factors for the induction of pluripotency [2], new evidence suggests that interactions between extracellular matrix proteins and integrin receptors play a role in controlling stem cell fate [1]. A functional role for integrin expression in cell niche has not been directly identified [3].

Integrins are a large family of heterodimeric, transmembrane cell surface molecules which serve as the main receptors for extracellular matrix (ECM) [4]. They provide for cell attachment, motility, and sensing of the extracellular environment. The different integrin heterodimers have been characterized for their specificity for different ECM proteins [5], and some integrins are known to have their affinity for ligand modulated by intracellular events, so called 'inside out' signaling. Responses to the ECM mediated by integrins, so called 'outside in' signaling, such as attachment dependent response to growth factors or avoidance of apoptosis [6], have also been demonstrated in a variety of model systems. Functional integrin receptors consist of an alpha and a beta subunit [7, 8], and five integrin heterodimers have been described as collagen receptors; $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha10\beta1$ and $\alpha11\beta1$ [9-12]. Expression of all these receptors except $\alpha10\beta1$ has been described in dermal fibroblasts, where they contribute to collagen type I binding [13] and collagen production [14].

Integrins localize in cell adhesion sites that are composed of a complex assembly of additional proteins and form a diverse and elaborate network [15]. The network in which

integrins have structural and molecular regulatory roles is known as the integrin adhesome [15], and consists of over 232 components. The majority of them (148 components) localize directly at the site of adhesion, while the remaining 84 components can associate with the adhesion transiently, and participate in subsequent signaling [16]. The integrin adhesome is a large, dynamic, and complex system that is not well characterized and still poorly understood.

At the tissue level, growth factors represent the main regulators of integrin expression. TGF β controls the transcription of numerous integrins in several cell types and tissues, and in most cases stimulates integrin expression. Induction of integrin expression by TGF β can be driven by cooperative signaling between the integrin and TGF β , thereby creating a forward loop [17]. TGF β not only regulates the expression of integrin ligands—including several members of the collagen family—but also stimulates the expression of integrin-associated proteins—including disabled 2, ILK, kindlin 1, paxillin and PINCH—which can also increase integrin activation [18]. Therefore, the transcriptional control exerted by TGF β can strongly affect integrin-mediated processes. Understanding the molecular mechanisms by which integrins mediate structural, biochemical and mechanical cues are involved in and subsequent determination of cell-fate is necessary.

Integrin α 11 is a major fibrillar collagen receptor in fibroblasts [19, 20], pairs with integrin β 1, binds with high affinity to collagen I [21], and is involved in myofibroblast differentiation and collagen reorganization [22]. α 11 expression has been described *in vitro* in human dermal fibroblasts [23] fibrosarcoma HT1080 [24], glioblastoma [25], and mouse embryonic fibroblasts [20, 26]. Transcription of integrin α 11 gene *ITGA11* is regulated by TGF β 1 via SMAD2/3, EST1, and SP1 transcription factors binding within the *ITGA11* proximal promoter [27] or by TGF β 2 via SMAD2/3 binding to a sequence in the *ITGA11* distal promoter [28].

FGF2 supplementation under low oxygen *in vitro* significantly alters fibroblast participation in wound healing, leading to a more pro-regenerative healing after cell transplantation *in vivo* [29]. Human dermal fibroblasts cultured under these conditions

significantly alter the expression of many genes, including activation of select stem cell genes [30].

In humans, five FGF2 isoforms can occur (18, 22, 22.5, 24 and 34 kDa), which display different subcellular localizations and contribute to different cellular behaviors [31, 32]. The 18 kDa FGF2 is known as the low molecular weight isoform, and signals predominately through the mitogen-activated protein kinase (MAPK) pathway [33]. Additionally, 18kDa FGF2 can also activate PI3K and PLC γ pathways. Combined, these pathways control cell type-specific signaling, and downstream effects include proliferation, growth, differentiation, migration, and survival [31, 34]. Additionally, the other isoforms of FGF2 are considered high molecular weight (HMW) and have intracellular effects on cells [32, 35]. NIH-3T3 cells, which were over-expressing LMW FGF2 (FGF2), were shown to modulate integrin expression, they increased $\alpha 5\beta 1$, and conversely, NIH-3T3 cells over-expressing HMW isoforms, down-regulated $\alpha 5\beta 1$. The observed increase in $\alpha 5\beta 1$ was shown to occur through increased rates of transcription, however both $\beta 1$ transcript was not increased, both $\alpha 5\beta 1$ protein increased [36, 37]. FGF2 has been observed to down-regulate $\alpha 11$ in mesenchymal stem cells as well as mouse embryonic fibroblasts, but the mechanism has not been described [22, 38].

When analyzing the changes in ECM and AM between hDF and iRC (Chapter 2), FGF2 was required for the shift. Additionally, FGF2 has been observed to down-regulate $\alpha 11$ in mesenchymal stem cells as well as mouse embryonic fibroblasts, but the mechanism has not been described [22, 38].

We have found that $\alpha 11$ is the most altered integrin in human dermal fibroblasts grown in low oxygen with FGF2 supplementation [39]. Here we describe the mechanism in the oxygen-mediated FGF2 effect on regulation of $\alpha 11$ expression in human dermal fibroblasts.

Decrease in expression of collagen-I binding integrins, in concert with decreased collagen-I expression and increased expression of collagen-I cleaving matrix metalloproteinase (MMP1) serve as an indication that FGF2-treated human dermal fibroblasts under low oxygen

conditions may be transitioning away from the production of a collagen-I rich niche. Understanding the molecular mechanisms by which integrin-mediated cellular phenotype is defined is necessary for many biological implications. Here we investigate the molecular mechanism of oxygen-mediated FGF2 effect on regulation of $\alpha 11$.

3.3 Results and Discussion

Down regulation of α 11: mRNA, protein, and localization

The observed loss of α 11 from iRC cells by RT-PCR (Figure 3.1a) and by Western blot (Figure 3.2b) led us to further characterize the loss of α 11. To determine a timeline for the down-regulation of *Itga11* transcript, samples were collected for qRT-PCR analysis every 24 hours for seven days after addition of FGF2. Down-regulation of α 11 was first observed 48 hours after the addition of FGF2, and transcript levels continued to decrease through day 7 (Figure 3.1c).

Next we asked if α 11 was being lost from focal adhesions where it is normally localized in hDF. Immunocytochemistry was used to determine if α 11 was being lost from focal adhesions in hDF compared to iRC. When compared, hDF showed the presence of α 11 in focal adhesions, while cells treated with exogenous FGF2 showed loss of α 11 protein from focal adhesions (Figure 3.1d).

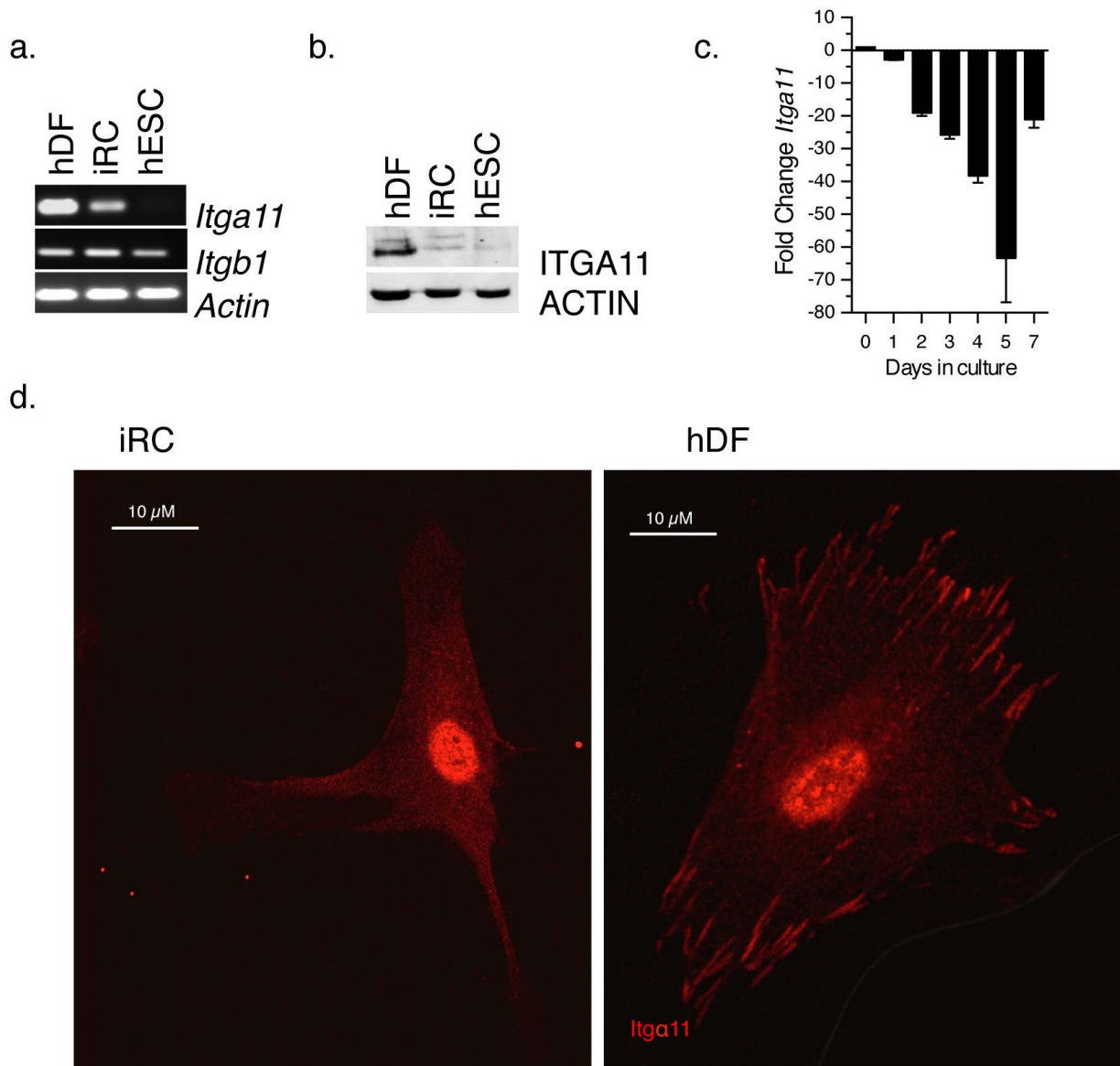


Figure 3.1 Expression localization and down regulation of integrin alpha-11

(A) RT-PCR for ITGA11, ITGB1 (positive control) and Actin on human dermal fibroblasts at 5% oxygen, with and without FGF2 (hDF and iRC respectively). (B) Western blots were probed with primary antibodies against Itg α 11 and actin. Protein was detected with HRP- conjugated secondary antibodies. hDF at 19% oxygen for 7 days were used as a positive control, and human embryonic stem cells as a negative control. iRC show a down-regulation of itg α 11 total protein when compared to control cells. Error bars indicate SD (n=2).

Comparison of *Itga11* transcript half-life and FGF2 treatment

In order to discern the mechanism by which FGF2 addition decreases α 11 expression several assays were used to understand the changes in expression at the transcriptional as well as translational level.

In an attempt to elucidate if FGF2 has a direct effect on the transcription of *Itga11*, we incubated hDF in FGF2 for 24, 48 and 72 hours as a control (Figure 3.2a), alpha-amanatin for 4, 8 and 24 hours (Figure 3.2b) and then measured the levels of transcript (Figure 3.2b, left half). In parallel we incubated hDF in FGF2 for the same amount of time in parallel. α -amanatin is an inhibitor of RNA polymerase II, and therefore should inhibit all new transcription. When comparing hDF in FGF2 and hDF with blocked transcription by 8 hours in α -amanatin, less *Itga11* transcript is present when compared to the same time point in FGF2 treatment, however after 24 hours the levels look comparable (Figure 3.2b). From this we conclude that addition of FGF2 to treated cells is capable of inhibiting new transcription of *Itga11* but not likely as a direct effect, as a direct effect would show similar rates to blocked transcription. Thus likely it is a downstream effect of FGF2 activation that inhibits new transcription of *Itga11*.

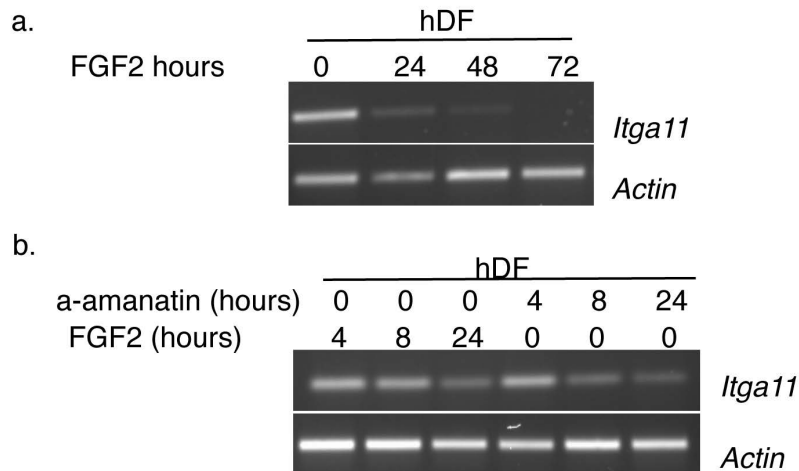


Figure 3.2 Comparison of effect of alpha-amanatin and FGF2 on levels of *Itga11* transcript

(a) Human dermal fibroblasts incubated in FGF2 for hours indicated, RT-PCR for ITGA11 transcript, actin used as loading control (b) RT-PCR for ITGA11 when cells were incubated in parallel in α -amanatin and FGF2 at time points indicated. Actin used as a loading control.

Methylation status of CpG islands

The methylation status of CpG islands within proximal promoter regions is known to regulate gene expression. Total genomic DNAs from hDF and iRC were digested with methylation-sensitive and methylation-dependent restriction enzymes and subjected to PCR with tiling primers that spanned two CpG islands within the ITGA11 gene (Figure 3.3a).

Genomic PCR with tiling primers spanning both CpG islands resulted in no PCR amplification for any of five regions when the DNA was digested with a methylation-sensitive restriction enzyme (HhaI whose cutting at CpG is blocked by methylation), and amplification of all five amplicons when the DNA was digested with a methylation-dependent enzyme (McrBc which cuts at methylated cytosines). This suggested that there was no apparent change in the methylation status of CpG islands when comparing cells expressing ITGA11 and cells not expressing ITGA11 (Figure 3.3b). Thus, the ITGA11 promoter is not methylated at the sites analyzed and the methylation status of the ITGA11 promoter showed no apparent change in its methylation status for the regions we amplified, and the FGF2-mediated down-regulation of α 11 likely is not due to promoter silencing through methylation.

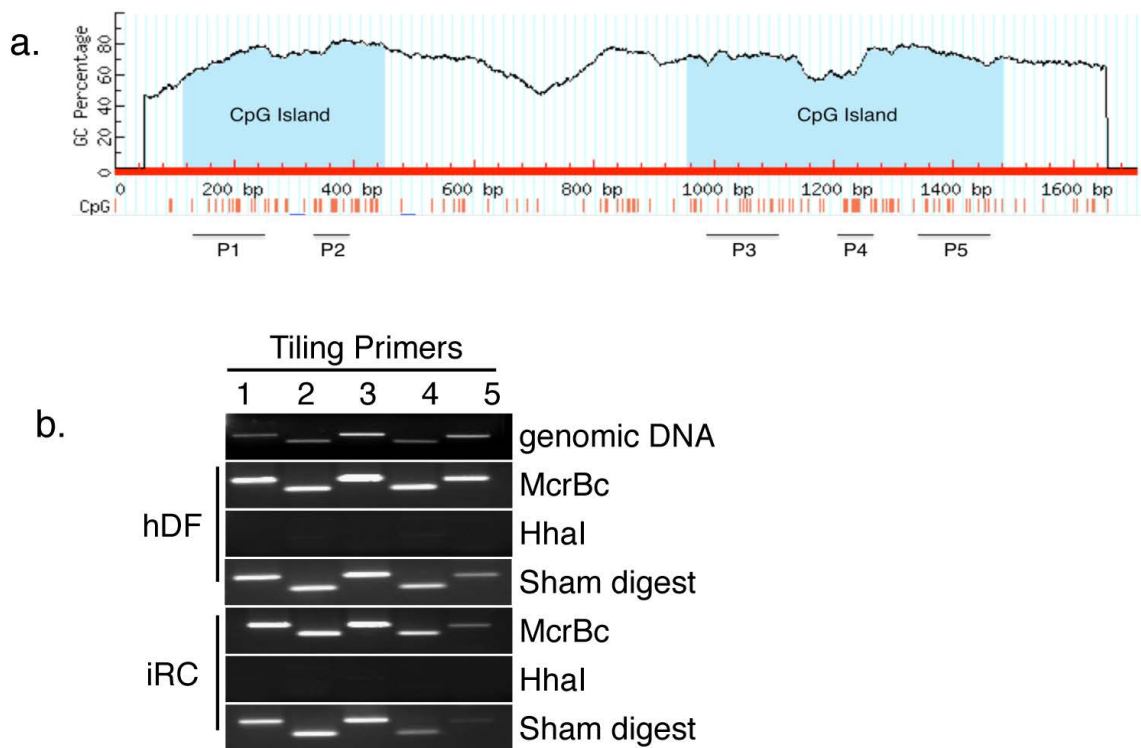


Figure 3.3 Effect of FGF2 on methylation of CpG islands in the ITGA11 promoter
(a) Genomic locations of CpG islands within ITGA11 promoter were identified using Transcriptional Regulatory Element Database and CpG islands were mapped using MethPrimer software. **(b)** PCR was performed with primers tiling two CpG islands mapped to primer sequences within the promoter region using NCBI Primer Blast. Control genomic DNA was used in addition to DNA from experimental treatments. DNA was subjected to digestion with a methylation-sensitive restriction enzyme (HhaI), methylation-dependent restriction enzyme (McrBc), or a sham digest was performed.

hsa-miR-29 levels after addition of FGF2

In a panel of TGF β -1-responsive fibrosis-associated genes α 11 was shown to be repressed by miR-29 through knock-down studies [40]. miR29 was shown to suppress TGF- β 1 up-regulated fibrosis-associated genes and, in knockdown, expression of fibrosis-related targets appear to have been repressed by miR-29. This suggests that miR-29a up-regulation suppresses the expression of α 11 [40]. Additionally, miR-29 is the only miR with sequence homology to *Itga11*, therefore making it a candidate for α 11 post-transcriptional regulation. To determine if the miR-29 family was regulating the expression of α 11 in hDF and iRC we investigated the level of total miR-29a and miR-29b in iRC's in comparison with hDF. Levels of miR-29 were assayed by qRT-PCR and no change was observed between hDF and iRC. qRT-PCR was used to analyze the expression levels of miR-29a and miR-29b in both hDF and iRC. Expression levels of miR29a remained consistent in hDF and iRC. Consistent levels of miR-29 in conjunction with loss of α 11 transcript suggested a non-miR-29-mediated gene silencing. (Figure 3.4).

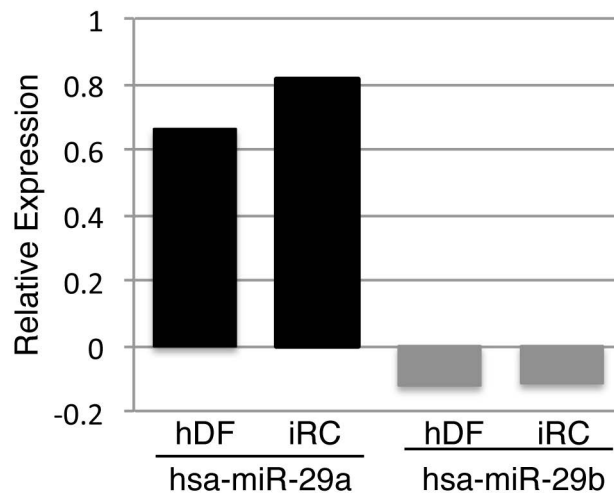


Figure 3.4 Effect of FGF2 on expression of hsa-miR-29a.

qRT-PCR reactions were performed using primers for hsa-miR-29a-3p and hsa-miR-29a-5p. Expression of hsa-miR-RNU6 was used for normalization. Expression levels were calculated using the delta delta-Ct method. hDF – fibroblasts grown at 5% oxygen, iRC - fibroblasts grown at 5% oxygen with FGF2.

TGF-β1 regulation of α11

TGF-β1 is a potent, dynamic regulator of ECM and AM expression, and α11 up-regulation has been shown to be dependent of TGF-β-mediated signaling [22, 28, 41]. TGF-β1 induces expression of α11 in primary fibroblasts through SMAD 2/3 and Sp1-mediated activation of the ITGA11 promoter (Figure 3.5) [41]. To determine if an increase in TGFβ-1 resulted in the down-regulation of α11 in our cells, we assayed the endogenous levels of TGFβ-1 in iRC cells when compared to hDF. The data (Figure 3.6a) showed that the increase in TGFβ-1 levels in iRC cells did not translate to an increase in α11 protein expression (Figure 3.6a). To assess if the presence of TGFβ-1 affected the expression of α11 in our cells, we treated hDF and iRC with exogenous TGFβ-1. A large range in TGFβ-1 concentrations was chosen to assess dose-dependence over a broad range of physiological concentrations. hDF were treated with increasing concentrations of TGFβ-1 in the absence of FGF2. Both low (0.05ng/mL-1.0ng/mL) and high (5ng/mL-50ng/mL) concentrations of TGFβ-1 induced an increase in α11 (Figure 3.6b).

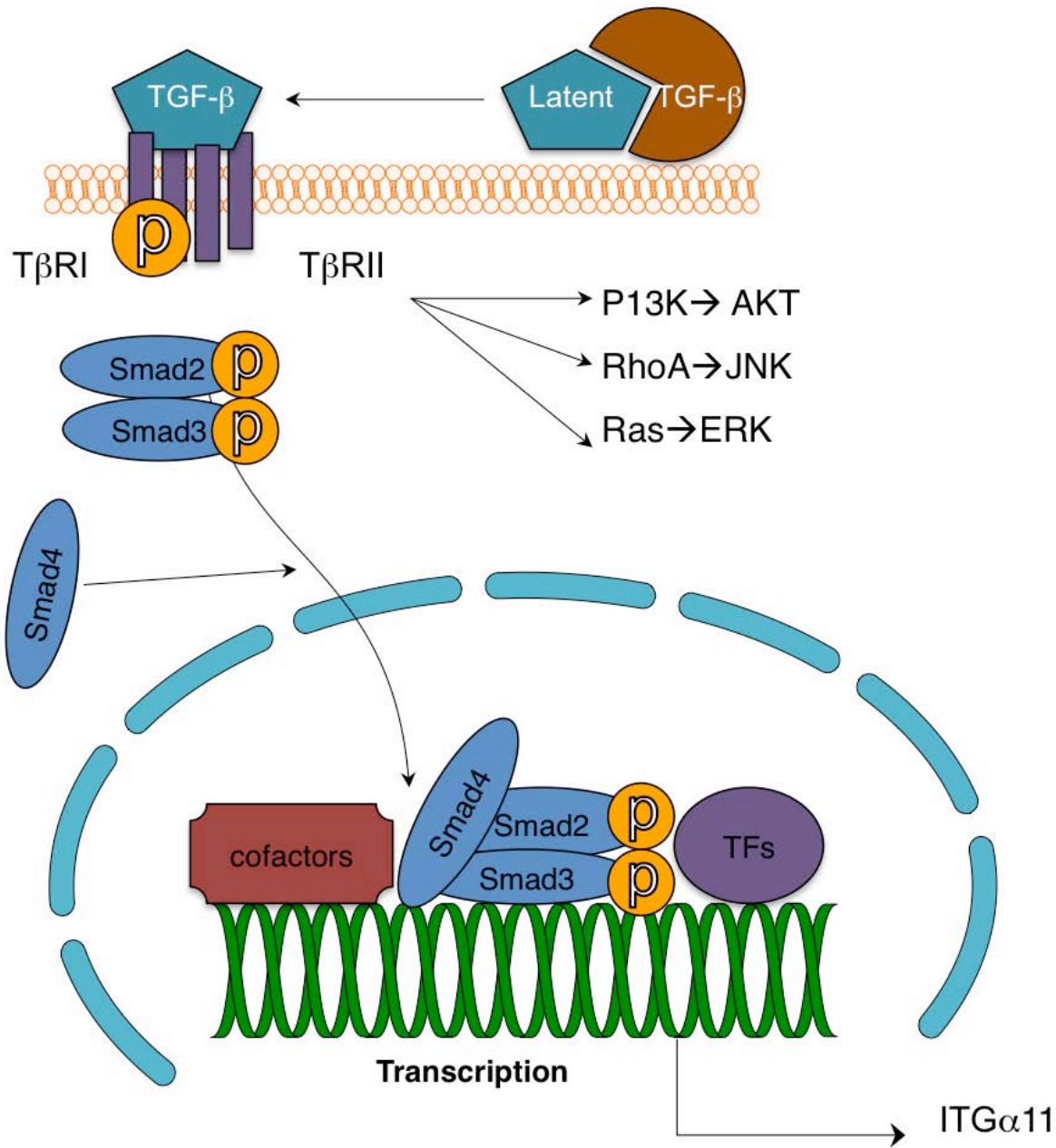


Figure 3.5 The transforming growth factor beta signaling pathway

Activation of latent TGFβ1 allows binding to TβRI, this binding results in the recruitment of TβRII which phosphorylates Smad2 and Smad3. Activated Smad2 and Smad3 heterodimerize with Smad4 and translocate to the nucleus. Smad2/3/4 complex act with cofactors and other transcription factors to induce or repress gene expression. Smad2/3/4 complex is known to induce expression of integrin alpha-11.

Due to the observation of an increase in TGF- β1 in iRC, and a loss of α11, we asked whether the FGF2 mediated loss in α11 occurs upstream of TGF-β1-mediated α11 expression. hDF and iRC were treated with three different concentrations of TGFβ-1 (0.1, 1.0, 10.0 ng/mL) in culture medium and assayed for α11 expression at day 7. Addition of different concentrations

of TGF β -1 in hDF and iRC resulted in a TGF β -1 dose-dependent down-regulation of α 11 expression and the presence of FGF2 strongly affected the signal (Figure 3.6c, right side). Cultures supplemented with TGF β -1 and treated with FGF2 showed a down-regulation of α 11 protein expression by Western blot (Figure 3.6c, right side) and was quantified using ImageJ (Figure 3.6d). These findings demonstrate that α 11 down-regulation as a result of exogenous FGF2 addition occurs upstream of TGF β -1 mediated regulation.

Additionally, to determine if other fibrotic associated molecules were involved with this transition, we looked at alpha-smooth muscle actin as another TGF β -1 mediated response factor and found that it responded similarly with FGF2 over-riding the TGF β -1-induced increase in alpha-smooth muscle actin (Figure 3.6e).

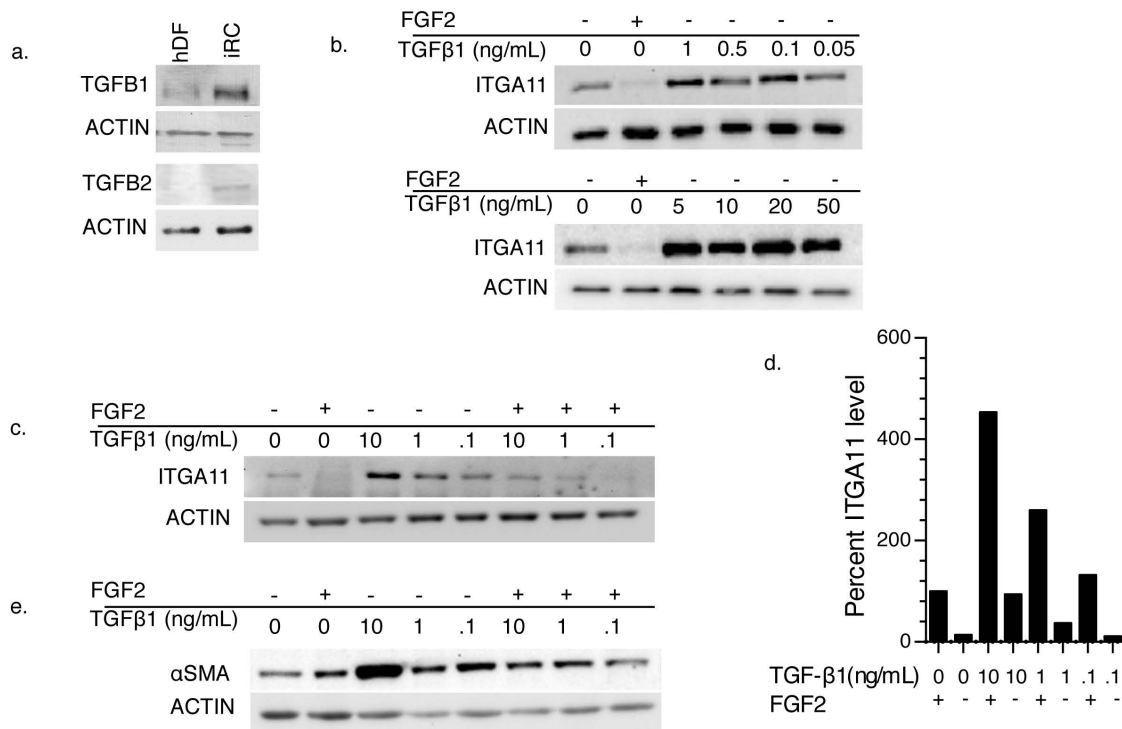


Figure 3.6 Effect of transforming growth factor TGF β 1 on expression of ITGA11.

(a) Total amounts of endogenous TGF β 1 and TGF β 2 in cells grown under different conditions. (b) Levels of ITGA11 in fibroblasts grown in low and high concentrations of exogenous TGF β 1 and (c) levels of ITGA11 in fibroblasts grown in low and high concentrations of exogenous TGF β 1 in the presence or absence of FGF2. (d) Quantification of ITGA11 expression by densitometry. (e) Expression of alpha smooth muscle actin (α SMA) in cells grown with TGF β 1 in the presence or absence of FGF2. Western blotting, actin was used as a loading control.

FGF2 mediated ERK1/2 regulation of $\alpha 11$ expression

The MAPK pathway involving ERK1/2 activation is a very robust pathway involved in many cellular processes and is also the canonical pathway for FGF2 signaling (Figure 3.7) [42]. Addition of exogenous FGF2 to the culture media resulted in phosphorylation of ERK1/2 kinase within 30 minutes of treatment (Figure 3.8a).

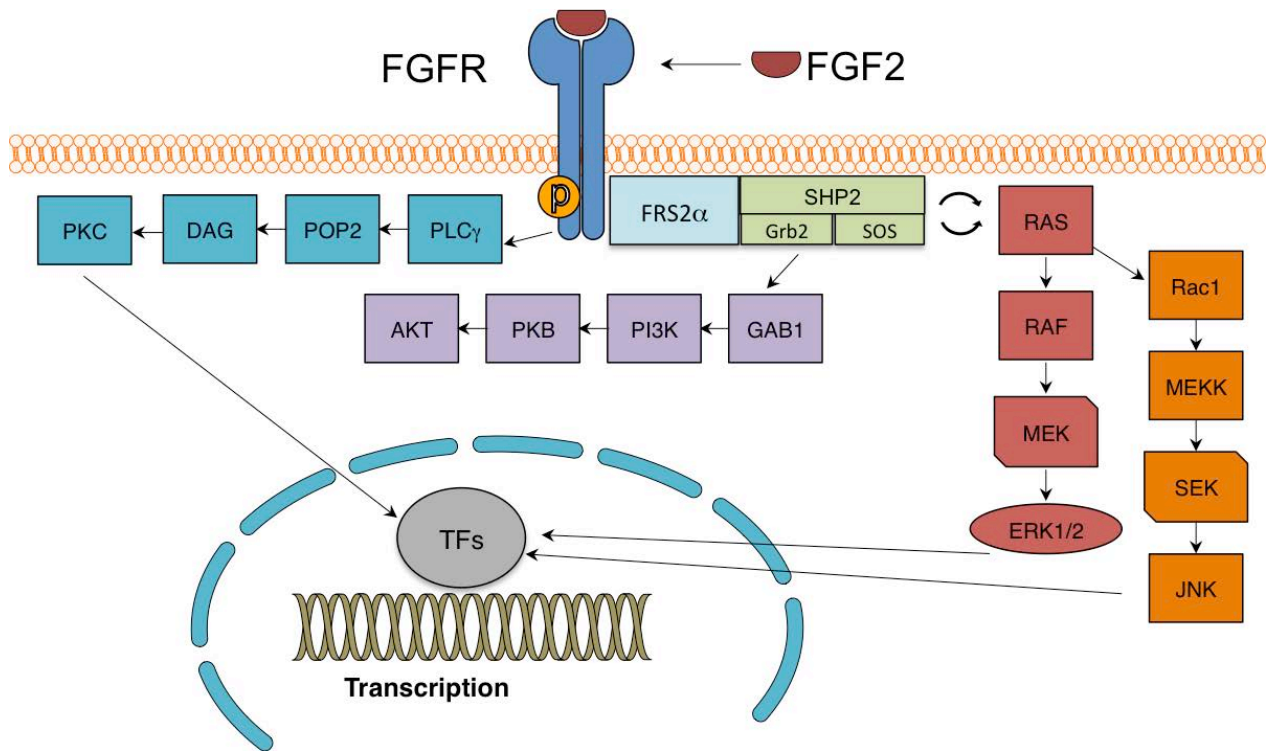


Figure 3.7 Basic fibroblast growth factor activation pathway

Binding of FGF2 to FGFR activates FRS2 through phosphorylation on several residues that facilitate the recruitment of the SOS and GRB2 adaptor proteins that trigger downstream activation of several kinase pathways effecting transcription.

To determine if ERK1/2 was involved in down-regulation of $\alpha 11$ expression upon addition of FGF2 we assayed ERK1/2 activation to determine its potential role in this process. FGF2 was added to human dermal fibroblasts that were grown under low oxygen conditions and treated with an ERK1/2 inhibitor (U0126) at a concentration that blocked ERK1/2 activation (Figure

3.8b). A concentration of 80µg/mL U0126 completely inhibited ERK1/2 activation. Western blot analysis showed that iRC in the presence of 80µg/mL ERK1/2 inhibitor, maintained expression of α11 at levels consistent with hDF that are grown in the absence of exogenous FGF2 (Figure 3.6b).

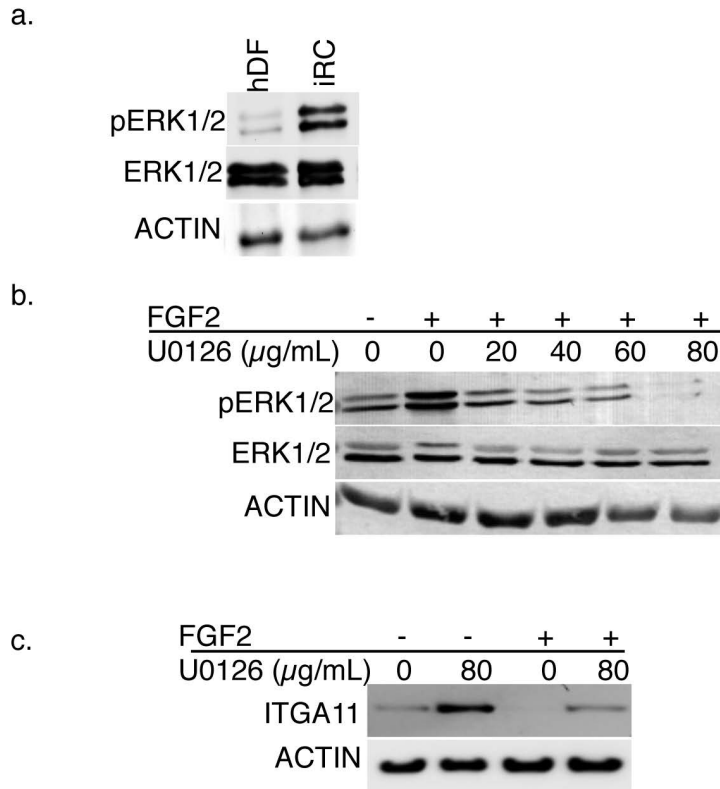


Figure 3.8 Effects FGF2-mediated ERK activation on expression of ITGA11.

(a) Levels of ERK (ERK1/2) and phosphorylated ERK (pERK1/2) kinase. (b) Levels of ERK1/2 and phospho-ERK1/2 kinase in the presence of increasing concentrations of ERK1/2 inhibitor U0126. Before assaying for ERK phosphorylation, cells were cultured without serum for 30 minutes. (c) Levels of ITGA11 in cells treated with 80µg/ml ERK inhibitor and FGF2. Western blotting, actin was used as a loading control.

Moreover, Western blot analysis of hDF treated with ERK1/2 inhibitor U0126 (with or without FGF2) showed increased α11 expression. These results support the hypothesis that ERK1/2 signaling is involved in the regulation of α11 expression. Notably, when ERK1/2 phosphorylation was blocked by U0126, iRC maintained higher α11 expression levels, indicating that activation of ERK1/2 was required for the down-regulation of α11 protein expression (Figure 3.8c).

3.4 Conclusions

Presentation of fibrosis in many organs is similar, regardless of initiation, and is mediated by the activation of fibroblasts into an activated fibroblastic phenotype. Therapy to reverse, halt or improve an existing fibrotic event is not available and still in clinical development [43]. Activated fibroblasts are thought to be largely responsible for the deposition of extracellular matrix [44]. We hypothesize that changing cellular signaling in the injury environment can modulate aberrant healing mechanisms, and scar formation from a typical fibrotic response should not remain the unfortunate outcome of the soft tissue wound healing. Regulation of cell fate through modulation of ECM suggests that extracellular matrix and integrin binding is involved in determining the cellular phenotype [1] yet, a functional role linking integrin expression profile with the cell fate has not been directly identified [3].

TGF- β 1 controls the transcription of several integrins throughout many different tissues and cell types. Additionally, in some cases the down-regulation of integrins has been reported via TGF- β 1 mediated signaling, however this is less often the case. TGF- β 1 mediated integrin expression in many cases can be driven by correlative signaling, such that integrin activation regulates its expression, as well as TGF- β 1 [17]. TGF- β 1 is a very well known regulator of many ECM molecules and integrin-associated proteins. Integrins are described as activators of TGF- β 1 as well as receptors for TGF- β 1 [45]. Taking all of this in sum, the relationship between TGF- β 1 and integrin mediated signaling is complex, however these pathways are involved in pathologies with fibrotic pathologies in cancer, wound-healing, and chronic fibrotic conditions [18].

Cutaneous wound healing in mammals proceeds through a sequence of several phases, including rapid production of TGF- β [46], immediate inflammatory response, cell proliferation and migration, and contraction and remodeling of ECM. Among various cell types that participate in the process, fibroblasts represent the most abundant cell type. Fibroblasts replace

the wound with granulation tissue composed of fibronectin and collagen-I. As the wound is re-vascularized, fibroblasts acquire an activated, highly contractile phenotype to close the wound with deposition of collagen-I dense scar [47]. In extreme cases when the routine healing process is compromised due to severe trauma, or deregulation of normal fibrotic/healing response irreversible fibrosis occurs resulting in deposition of excessive fibrous connective tissue and dense scar formation. Pathological fibrosis is regulated by ECM secreting activated-fibroblasts and accompanies the progression of many chronic inflammatory diseases. Normal scar reorganization involves the remodeling of initially deposited collagen-I followed by full or partial restoration of prior tissue function, however fibrotic pathologies compromise the function of affected organs and tissues. Irreversible fibrosis is responsible for death from end-stage liver, kidney, heart diseases, idiopathic pulmonary fibrosis [44] and unresolved fibrotic conditions are features of many autoimmune diseases [48]. TGF- β 1 mediates the fibrotic response through activation of ECM genes [49], and suppression of ECM regulators by degradation [50]. In fibrotic conditions, TGF- β 1 induced Smad3 signaling results in deposition of large amounts of collagen-I by activated-fibroblasts [51, 52].

FGF-2 has a robust myogenic effect on fibroblasts, and its participation has been linked to multiple fibrotic disorders in oral submucosa [53], pulmonary fibrosis [54, 55] and renal fibrosis [56] to name a few. These studies allow us to draw the conclusion that FGF2 can be induced by TGF- β , which creates a complex relationship between these signaling molecules [56, 57].

To add to this broad range of effects, FGF-2 has also been shown to inhibit TGF- β induced myofibroblast differentiation [58-62] and in cases of non-pathological wound healing, it has been shown to be able to induce apoptosis in myofibroblasts, but not in resident fibroblasts which have not taken on a myofibroblast phenotype [62, 63]. These studies indicate that FGF2 has regulatory roles in mediating the balance between scar and tissue formation in wound healing, as it triggers proliferation of fibroblasts, yet keeps myofibroblast populations limited through

apoptosis. The role of FGF2 signaling in fibrosis remains unclear and continues to get increasingly complex, as the regulatory loop connecting TGF- β 1 mediated fibrotic conditions and FGF2 has yet to be fully defined.

FGF2 has been shown to be involved in the promotion of several tumor types through activating proliferation pathways, and aiding in survival of cancer cells by inducing vascularization [64]. The relationship between FGF2 and cancer is not easy to elucidate, as FGF2 has also been shown to have a role in tumor suppression [64].

Integrin α 11 has been described as a contributing factor to fibrotic tumor stroma [65]. In many cases fibrotic tumors impermeable cytotoxic chemotherapy because they are high density [66]. Integrin- α 11 contributes to this dense environment through high levels of binding to collagen-I [65]. Mitigation of α 11 binding through a pathway that can override TGF β -1 signaling could provide therapeutic targets for dense fibrotic tumor stroma.

Understanding the molecular mechanisms by which integrin-mediated cellular phenotype is defined is necessary, and would have a wide range of biological implications. Fibrosis research is focused on elucidating molecular mechanisms that can terminate irreversible fibrosis [18]. Any progress in the development of a treatment using a common tissue fibrosis pathway, especially one that can reduce TGF β -1 fibrotic conditions, could result in the identification of potential therapeutic targets for common cellular pathways.

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Chapter 4

Mesenchymal-to-Epithelial Transition in Induced Regeneration Competent Cells—Investigating a role for Integrin Alpha-11

4.0 Abstract

Advancements in cellular therapy for regenerative medicine currently are limited by clinically non-compliant cell sources. To this end, the importance of understanding the molecular mechanisms underlying the cell-fate conversion and cell-fate reversal for development of autologous cell sources is becoming increasingly clear. Here we describe changes in the human dermal fibroblasts (hDF) undergoing induced regeneration competence (iRC), a process that is defined by lowered oxygen with exogenous FGF2 supplementation, and characterized by the induction of stem cell gene expression and increased cellular life span *in vitro*. In this chapter we characterize the presence of focal adhesions and focal adhesion kinase activity, and lay the groundwork **to support the hypothesis that that mesenchymal-to-epithelial transition (MET) may be mediating a shift in the extracellular matrix and adhesion molecule profile**. We identify early MET transcriptional targets, confirm that our treated cells are in the initiation stages of MET, and try to get a complete transition accomplished via a 3D cell culture protocol. While complete MET is not achieved using our culture conditions alone, the partial transition we observe may lead to improved trans-differentiation protocols, and ultimately result in the development of clinically compliant cells sources.

Objectives and Hypothesis

This part of my work aims to determine if the transition from a fibroblast to an induced regeneration competent cell, induced by our culture conditions, proceeds through MET, and to demonstrate if this transition is a consequence of down-regulation of $\alpha 11$ expression.

4.1 Introduction

Study of cell-fate conversion through the induction of pluripotency is a critical model system, however progress towards defining methods for generation of an autologous, FDA compliant cell source for clinical applications is still necessary. We have developed a novel approach that is characterized by the induction of stem cell gene expression and increased cellular life span in human dermal fibroblasts *in vitro*. Our system does not utilize the use of transgenes, and is characterized by use of defined culture conditions involving culturing cells at 5% O₂ and supplementation with FGF2 [1]. Understanding the molecular mechanisms underlying the cell-fate conversion observed in hDF grown in 5% O₂ with FGF2 (iRC) and cell-fate reversal is important for the development of autologous cell sources as therapeutics, while maintaining a clinically compliant derivation protocol. Cells undergoing a MET transition show some features similar to iRC cells. For this reason we decided to study the molecular mechanisms of MET in order to determine if MET is the mechanism by which our defined culture conditions activate stem cell gene expression in hDF.

Induced pluripotent stem cell (iPSC) derivation methods gave what were once thought terminally differentiated cells [2, 3] the potential to be autologous cell sources for a wide variety of applications, however to date there have been no examples to demonstrate that reprogrammed cells are clinically compliant. Initial iPSC screens looked at changes in epigenetic state, as well as transcriptional changes throughout the reprogramming process and one of the discoveries showed that mesenchymal-to-epithelial transition was a requirement for reprogramming of somatic cells to a pluripotent state [4, 5]. EMT and its reverse, MET are characterized by changes in the extracellular matrix (ECM) and subsequently the adhesion molecule (AM) expression profile. During EMT, epithelial cells must break down the basement membrane components, and modify attachment substrates in order to attach to a distinct niche [6]. This has been demonstrated by studies focused on Rho-mediated basement membrane

breakdown which initiates early-stage developmental EMT [7]. Moreover, altered expression of metalloproteases [8], cytoskeletal proteins [9], collagens [10] and integrins [11] have all been linked to EMT and MET.

ECM is an assembly of fibrous proteins, (i.e. collagen, elastin, fibronectin, laminin) and proteoglycans, (i.e. heparan sulfate, chondroitin sulfate), and each tissue has a specific ECM composition [12]. ECM was initially thought to serve as a scaffold to maintain tissue and organ structure, but is now known to be involved in regulation of cell behavior including cell proliferation, growth, survival, morphology, migration, and differentiation [13]. Tissue-specific ECM remodeling through degradation and reassembly is constant, and that can be triggered and regulated by activation of receptors such as integrins and syndecans [14]. ECM remodeling rates are elevated during specialized times, such as EMT and its reverse MET [14].

One of the first noticeable changes during the reprogramming of fibroblasts is a morphological transformation that is characterized by tightly packed clusters of rounded cells, resembling MET characteristics [3, 15]. This is not surprising, since throughout the development, EMT/MET are frequent and about ten percent of the human genome is differentially regulated during these transitions [16]. Moreover, major changes in miRNA profiles of cells undergoing EMT/MET have been observed in the transition. When describing EMT, down-regulation of E-cadherin and up-regulation of Snail are major regulators and are hallmarks of this transition, additionally, the reverse can be said for MET [17]. The relationship between these proteins is not complex, however the downstream effects are. Snail is a transcription factor that suppresses the transcription of E-cadherin as well as other molecules that are responsible for epithelial phenotype [18, 19]. E-cadherin is a transmembrane protein within adherence junctions in epithelial cells that maintains cell-cell contact [20].

When studying either gene expression profiles [21] or morphological changes [17] of MEFs during the initial stages of reprogramming to iPSC, there were similarities to MET. This pattern included the up-regulation of epithelial genes as well as the down-regulation of key

mesenchymal genes. Based on the observed gene expression dynamics three sequential phases of reprogramming were identified: initiation, maturation, and stabilization [21]. Changes in MET-associated transcript levels were evident when cells were still dependent on exogenously supplement reprogramming factors, and occurred before the cells activated an embryonic gene expression program of their own [21]. This suggests that MET may be one of the earliest changes fibroblasts undergo during cellular reprogramming. Reprogramming of mouse fibroblasts to iPSC involves a switch from a mesenchymal to an epithelial transcriptional program and changes in cell morphology [17, 21]. Transcription factors used to reprogram MEFs were shown to down-regulate mesenchymal genes and activate epithelial associated genes. Of these genes, E-cadherin has also been implicated as a regulator and a potent controller of human embryonic stem cell pluripotency (hESC). Stabilization of E-cadherin through Rho-associated kinase inhibitor (ROCK) has been shown to improve overall viability in hESC colony formation during passaging [22-25]. Improving our understanding of the molecular steps of reprogramming could lead to improvements and insights into how cellular identity is reversed.

4.2 Results and Discussion

Focal Adhesion Dynamics

Vinculin is a focal adhesion protein involved in the regulation and structure of focal plaques, and allows visualization of all focal adhesions. To determine if iRC cells, which were shown to significantly down-regulate the expression of integrin alpha-11 and collagen-I, were remaining adherent, we looked at overall attachment through staining for vinculin. Focal plaques were present in iRC and hDF, with iRC appearing to have more focal adhesions by comparison of images (Figure 4.1). In order to determine if signaling may be changing through focal adhesion kinase (FAK) pathway, RT-PCR for FAK downstream signaling molecules was used as an initial screen to identify changes (Figure 4.2a). After no changes were seen in the transcript levels of FAK pathway members, FAK phosphorylation status was determined through Western blotting of iRC and hDF using hESC as a control (Figure 4.2b).

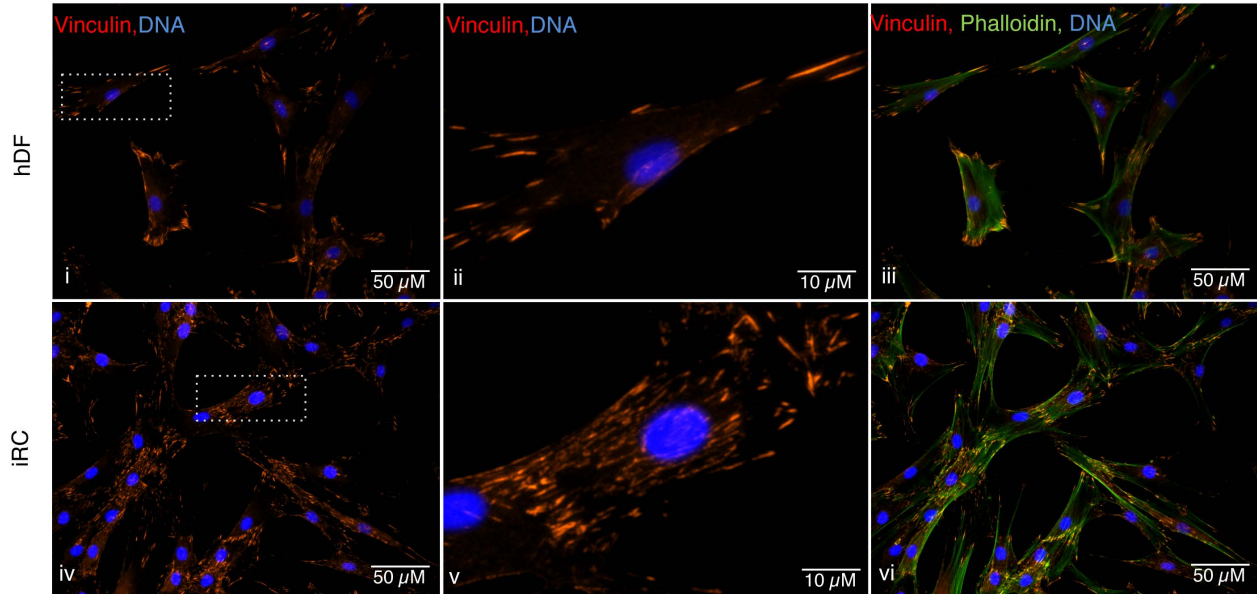


Figure 4.1 Vinculin immunocytochemistry

Immunocytochemistry of fibroblasts labeled with primary antibodies against vinculin and detected with secondary antibodies conjugated to AlexaFluor568 (red); and phalloidin conjugated to AlexaFluor488 for visualization of actin (green).

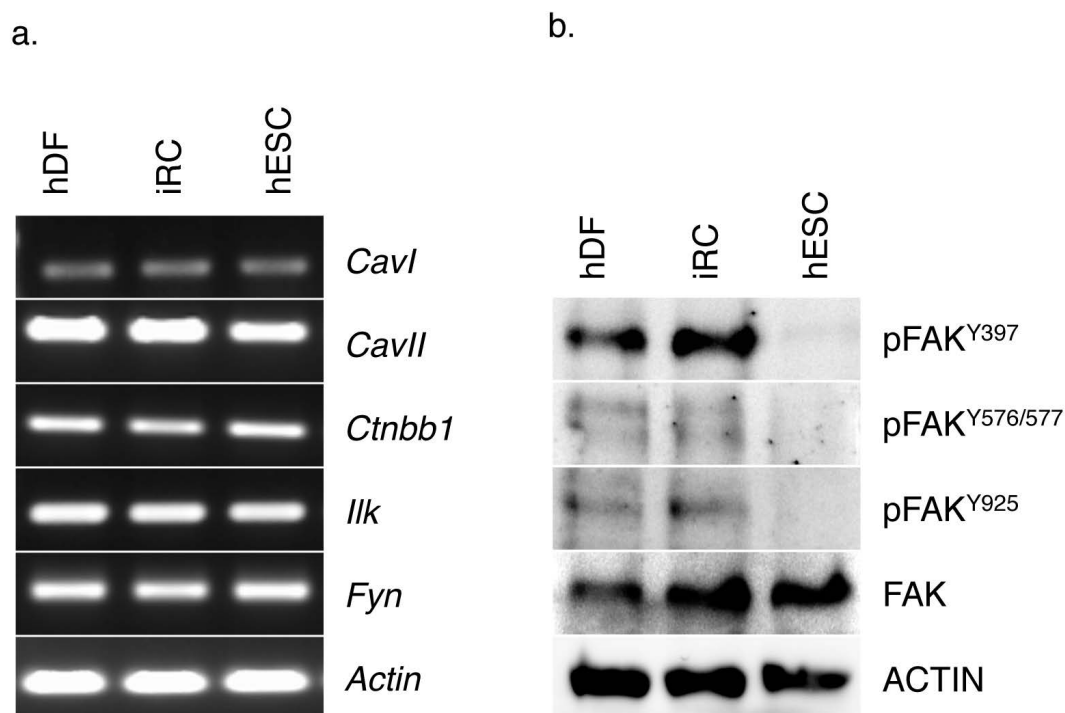


Figure 4.2 Focal Adhesion Kinase Signaling

(a) RT-PCR for focal adhesion kinase associated genes in iRC and hDF cells hESC used as a positive control for RT-PCR and actin used as a loading control. (b) Western blot analysis of focal adhesion kinase FAK. Actin was used as a loading control.

FAK is a non-receptor protein tyrosine kinase, which is associated with integrin-mediated signaling through the FAK-Src complex which regulates mobility, survival and cell spreading [26]. Activation of FAK by phosphorylation through integrin clustering leads to auto-phosphorylation of FAK at tyrosine-397, which serves as a binding site for Src family kinases P13K and PLC γ . Src family kinases phosphorylate FAK in the catalytic domain at tyrosine-576/577, and in the c-terminal region at tyrosine-925 [27]. Activating phosphorylation of FAK at tyrosine-397, tyrosine-576/577 and tyrosine-925 were present at comparable levels in hDF and iRC (Figure 4.2b). Activating auto-phosphorylation (Y-397) occupied the largest percentage of phosphorylation out of total FAK present in both hDF and iRC, with elevated levels of total FAK in iRC. Secondary phosphorylation residues (Y-576/577, Y-925) had comparable levels of phosphorylation (Figure 4.3b). Elevated levels of total FAK in iRC (Figure 4.2b), presence of large numbers of focal plaques (Figure 4.1), and transcriptional changes in other integrin

subunits (Chapter 2) suggested that alternate α and/or β subunits and alternate extracellular matrix substrates are being expressed substituting for $\alpha11\beta1$ collagen-I mediated attachment.

$\alpha6\beta1$ and $\alpha6\beta4$ are receptors for laminins predominately present in basement membrane [28]. $\alpha v\beta3$, $\alpha5\beta1$ are RGD integrin receptors, as well as receptors for fibronectin [29] and vitronectin [30]. However, when comparing potential pairs for all up-regulated α and β subunits, the substrates being bound are likely laminins, RGD receptors binding pairs, fibronectin, and vitronectin. It is important to point out the different receptor binding pairs because signal transduction pathways triggered by differential binding of ECM have different effects on cell behavior, such as proliferation, survival/apoptosis, shape, polarity, motility, gene expression and differentiation.

Mesenchymal-to-Epithelial Transition

Table 4.1 Genes deregulated by mesenchymal-to-epithelial transition

Table 1: MET genes for qRT-PCR analysis

Genes down-regulated during MET	<i>Ncad</i>	calcium dependent cell-cell adhesion glycoprotein
	<i>Slug</i>	Transcriptional repressor. Involved in the generation and migration of neural crest cells
	<i>Snail</i>	binds E-cadherin gene promoter and represses its transcription
Genes up-regulated during MET	<i>Rex1</i>	involved in self-renewal in ES cells
	<i>Dsp</i>	necessary for desmosomes, tight junctions
	<i>ECad</i>	epithelial specific, cell-cell adhesion glycoprotein
	<i>Spp1</i>	binds $\alpha v\beta3$ and $\alpha5\beta1$ integrins and induces focal adhesion assembly

Classic EMT is associated with the relocation of cells from a basement membrane microenvironment into a fibrillar ECM, and MET can be characterized by the opposite [31]. An observed change in expression levels of integrins subunits are often an indication of a change in cell to ECM interaction [32]. Additionally, integrin signaling has been described to facilitate the

transition during EMT, as various integrin expression profiles are specific to both epithelial and mesenchymal phenotypes [33].

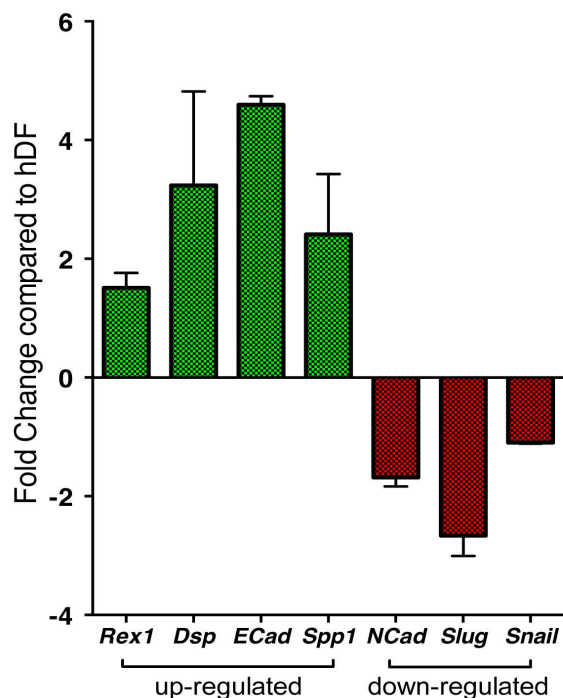


Figure 4.3 qRT-PCR for MET genes qRT-PCR for on human dermal fibroblasts, at 5% oxygen with and without exogenous FGF2 (hDF and IRC respectively) genes indicated in MET transition. Targets in red are indicative of genes that should be down regulated during MET, genes in green are indicative of genes that should be up regulated.

After observing a change in integrin subunits, and our previous findings that hDF were no longer attaching to collagen-I and more likely were making a basement membrane substrate (Chapter 2), we wanted to determine if iRC were undergoing a cell-fate conversion towards a more epithelial phenotype via MET. We analyzed the gene expression profiles of iRC for genes that were associated with the initial stages of MET (Table 4.1). To pick biologically relevant targets, we surveyed the Human EMT RT2 Profiler™ PCR Array (SA Biosciences) to identify a gene ontology specific to MET and identified significantly up-regulated and down-regulated genes from the subset of significantly up or down-regulated genes in the transcriptome array

[34]. qRT-PCR for selected MET associated genes (Table 4.1) on hDF, iRC were assayed to determine if the shift in ECM/AM we were observing was potentially a consequence of MET.

Human dermal fibroblasts were cultured under the previously described defined conditions for 30 days, as this was the time point when stem cell gene expression had been previously described, and we expected the most robust phenotype change to take place [1]. Analysis by qRT-PCR showed an overall shift in the expression of all assayed MET associated genes in iRC (Figure 4.3). Consistent with expected gene expression changes during MET, we observed an up-regulation in *Rex1*, *Dsp*, *Ecad*, *Spp1*, and a down-regulation in, *Ncad*, *Slug*, and *Snail*, suggesting that iRC at day 30 were in the initiation stage of MET.

Epithelial Cadherin Expression

To determine if iRC had passed through MET after 30 days of culture, iRC cells were analyzed by Western blot for E-cadherin (ECAD) expression, as the shift from N-cadherin (NCAD) to ECAD is considered to be a defining characteristic of cells that have completed MET. iRC grown for 30 days on tissue culture plastic, did not show expression of ECAD by Western blot when compared to hESC positive controls (Figure 4.4a) .

Previous work on regeneration had shown that a MET transition was fundamental to the *in vitro* generation of neuro-epithelium, and it was necessary for cells to be cultured in 3D in order for this transition to occur [35]. To overcome the challenges associated with lack of ECAD protein expression under our experimental culture conditions, and based on previous studies demonstrating the importance of the 3D environment, we redesigned the experiment using 3D aggregation culture of iRC and hDF (Figure 4.4b). However, comparison of ECAD protein expression between the 2D cultures with 3D cultures of iRC and hDF did not demonstrate differences in protein expression when compared with positive controls (Figure 4.4a). To confirm the Western blot results and eliminate the possibility that the Western blot detection limit was not producing false negatives, immunohistochemistry was used to analyze ECAD protein

expression in our 3D aggregates (Figure 4.4b). However, the immunocytochemistry results remained consistent with the Western blot results demonstrating no ECAD expression in our aggregates (Figure 4.5b).

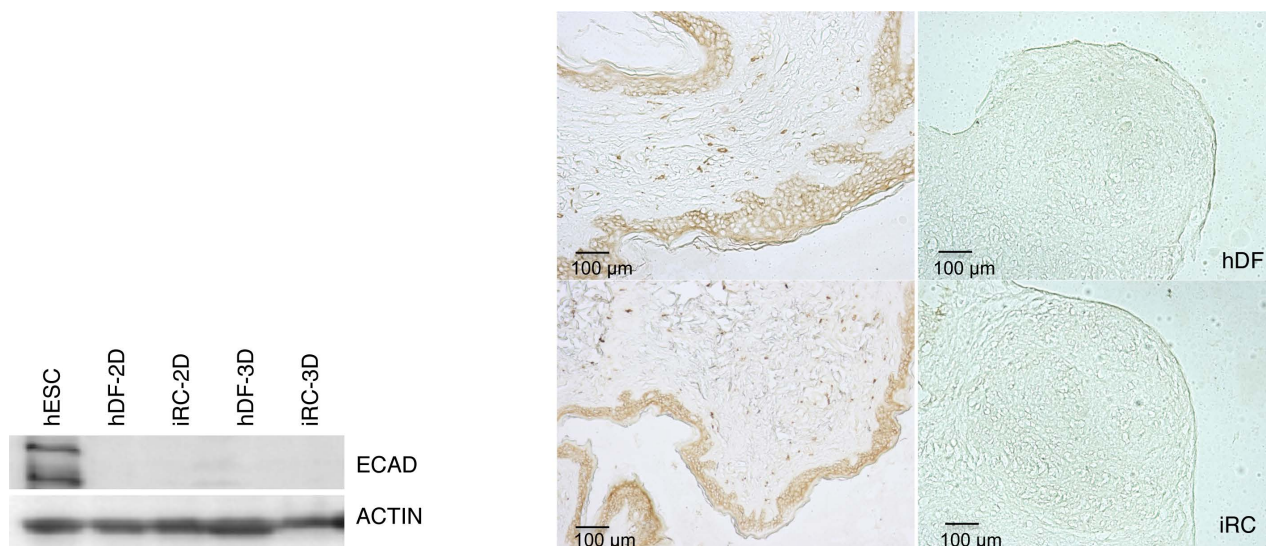


Figure 4.3 Induction of Mesenchymal-to-Epithelial transition (MET) via aggregation.

Total amounts of epithelial cadherin (ECAD) was measured in hDF and iRC cells grown in 2D and 3D by Western blot. hESC were used as a positive control for ECAD, and actin was used as a loading control. (b) Immunohistochemistry for ECAD in hDF and iRC grown in aggregates, protein visualization done with DAB staining. Human skin was used as a positive control for ECAD staining.

Over-expression of Integrin alpha-11

Expression of ECAD is a characteristic of late stage MET. As a result of lack of ECAD expression, we hypothesized that our iRC cells could be in the initiation phase of MET. Based on our previous findings showing $\alpha 11$ to be the most highly deregulated ECM/AM molecule out of all targets analyzed (Chapter 2), our next step was to elucidate if there was a potential role for the down-regulation of $\alpha 11$ in the acquisition of more epithelial phenotype. An over-expression $\alpha 11$ construct containing a c-terminal GFP tag was built. Restriction digestion analysis confirmed that clones #6 and #12 contained all inserts in the correct orientation (Figure 4.5). For validation of $\alpha 11$ -GFP construct (Figure 4.5a) clones #6 and #12 were expressed in ChoK1 and

HeLa cells. RT-PCR for $\alpha 11$ was done in ChoK1 cells for clone #6 (Figure 4.6b). Cells transfected with $\alpha 11$ -GFP were positive for *Itga11* transcript, and interestingly *Itgb1* as well.

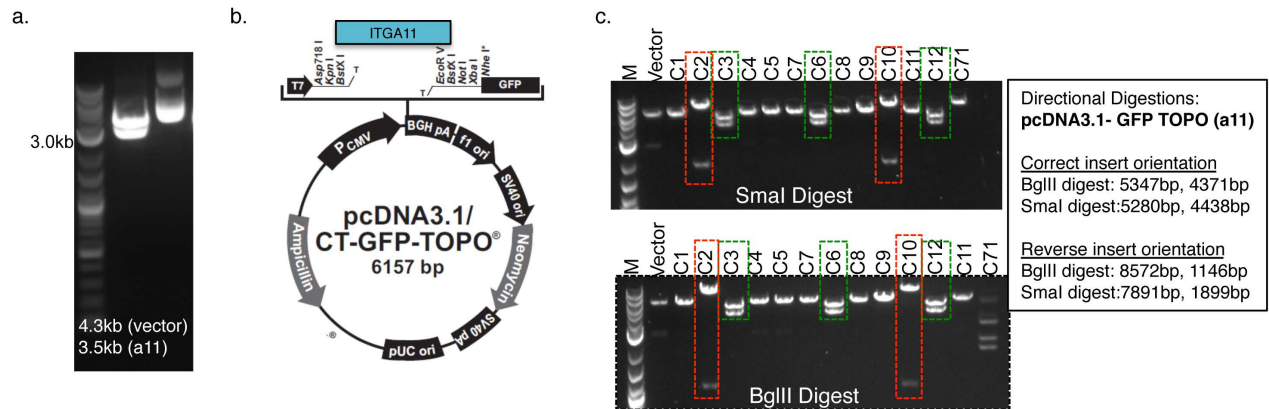


Figure 4.4. Cloning of *Itga11*-CT-GFP

(a) pENTR233.1-sfil-a11 plasmid digested with *SfiI* enzyme to isolate *Itga11* from plasmid. Lane 1—2 log ladder, Lane 2—pENTR233.1-sfil-a11 plasmid digest, Lane 3—uncut pENTR233.1-sfil-a11 control. (b) plasmid map for pcDNA3.1/CT-GFP-TOPO used for blunt ligation of *Itga11* into multiple cloning site. (c) Directional digests to determine insert orientation in clones 1-13, digests marked in red indicate ligations inserted backwards, digests marked in green indicate clones inserted in the correct orientation.

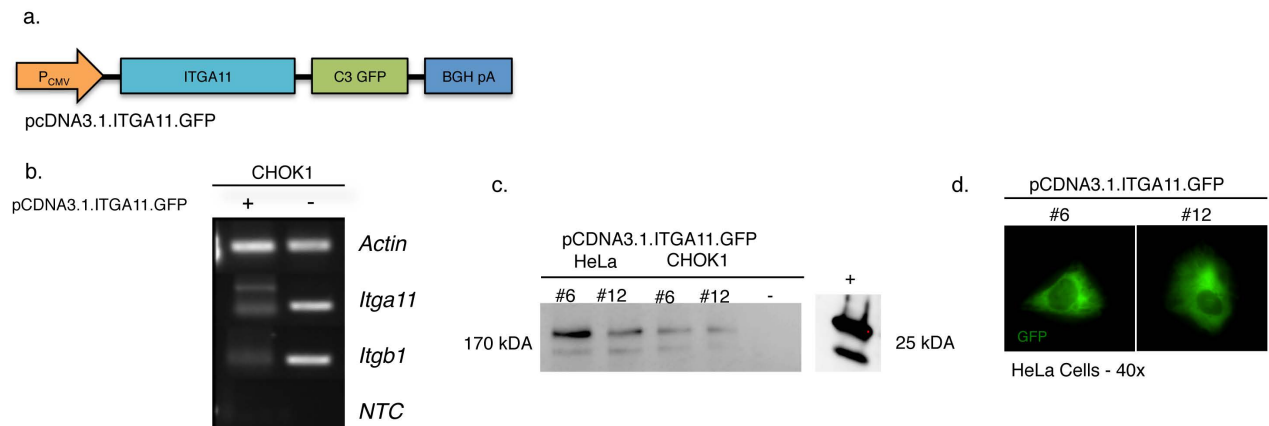


Figure 4.5 Over-expression of *Itga11*-CT-GFP

(a) pcDNA3.1-*itga11.ctGFP* construct map (b) HeLa and CHO-K1 cells were transfected with pcDNA3.1-*Itga11.gfp* clones, and cell lysates were probed for protein by Western blot with anti-integrin alpha 11 antibody. HeLa cells were live imaged at 40x.

This suggests that $\alpha 11$ somehow over-expression of $\alpha 11$ may be regulating the expression of *Itgb1* transcript, however as we were interested in the role of $\alpha 11$, we did not follow this result (Figure 6a). In order to determine if we were making full-length protein, we ran Western blot analysis with anti-GFP primary antibody (Figure 4.6c). Also fluorescence imaging of HeLa cells confirmed expression and localization of $\alpha 11$ -GFP (Figure 4.6d).

The transfection efficiency in the HeLa and CHOK1 cells was low, both with lipid-based and electroporation-based transfection methods. The overall transfection efficiency we saw in these transformed lines was around 10-15% based on quantification of GFP-positive cells. Transfection attempts on hDF cells using lipofectamine as well as electroporation did not result in positively transfected cells. This could be as a result of hDF being a very difficult cell type to transfect combined with the relatively large plasmid size of 9.6kb. Given the limited population doubling number that hDF cells can undergo, it was not feasible to create a stably transfected cell line as hDF reach senescence before a stably transfected population could be established

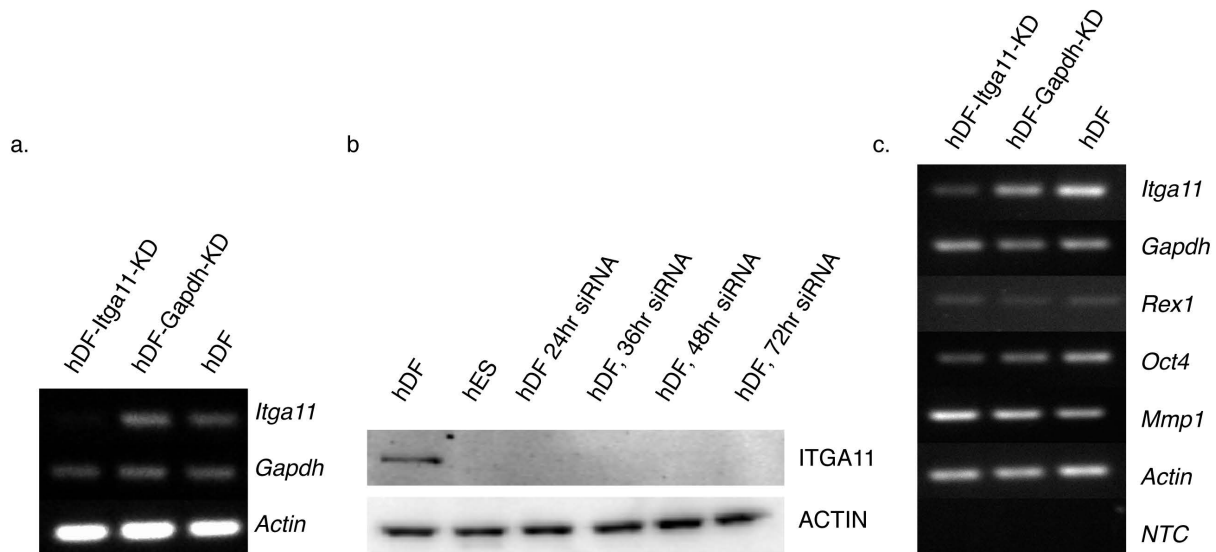


Figure 4.6 Knock-down of *Itga11*

(a) RT-PCR for knock-down of *Itga11* and *Gapdh* in hDF. (b) Western blot for $\alpha 11$ in hDF at 24, 48 and 72 hours post transfection with siRNA. hESC used as a negative control, hDF used as a positive control. Actin was used as a loading control for Western blotting. (c) RT-PCR for genes associated with knockdown of $\alpha 11$, Actin used as a loading control, NTC indicates no template control.

so the over-expression of $\alpha 11$ could not be studied in hDF cells.

Knock-down of Integrin alpha-11

Due to the inability to create a stably transfected hDF line, an alternative route was followed to show the effect of lack of $\alpha 11$ expression. Transient knock-down experiments were performed in hDF using siRNA to *Itga11* (Figure 4.7a). Using a commercially available siRNA against human $\alpha 11$ (ON-TARGETplus siRNA, Dharmacon, GE Healthcare) *Itga11* was transiently knocked down in hDF. Transcript knock-down was validated by RT-PCR (Figure 4.7a), and protein level was analyzed by Western blot (Figure 4.7b). Knock-down of $\alpha 11$ protein was confirmed as early as 24-hours and as late as 72-hours, and the knock-down hDF were assayed for changes in expression levels of *Itga11*, *Gapdh*, *Rex1*, *Oct4*, *Mmp1* (Figure 4.7c). No major changes were observed in the five genes of the genes at 72-hours post knock down. *Rex1* and *Oct4* were chosen as early markers of both our defined culture system as well as MET. Additionally *Mmp1* expression levels were analyzed as it was known that *Mmp1* is transcriptionally up-regulated when cells are cultured using our defined culture conditions. No change in gene expression profile of hDF was seen after 72 hours of $\alpha 11$ knock-down. This may be due to the short transient knock-down (Figure 4.7d).

4.3 Conclusions

Culturing hDF under our experimental culture conditions resulted in a shift away from collagen-I expression in iRC, however that did not appear to have an effect on overall cellular attachment as the vimentin-containing focal adhesions stained strongly, and the focal adhesion kinase (FAK) pathway remained active. qRT-PCR confirmed the up-regulation of several integrin α -subunits associated with attachment to ECM components present in basement membrane, and showed an up-regulation in gene expression that was consistent with MET.

It was not possible to determine if integrin mediated signaling, through integrin alpha-11 (α 11) had a role in the MET associated transcriptional shift observed in iRC as the overexpression construct used did not express α 11-GFP in hDF.

Lack of ECAD expression a marker for late MET could be due to many factors, as MET requires a very complex set of molecular events, and it is likely that iRC have not undergone a complete transition. The observed gene expression dynamics consisting of three consecutive phases of reprogramming suggest that iRC may be in an early (*initiation*) phase of MET and in order for a full MET to occur, iRC might require exogenous forced factor expression in order to cross the threshold. Additionally, REX1 protein was not detectable by Western blot at day 30 and likely a contributing reason to why iRC could not move into the stabilization phase of MET even in 3D culture. Previous work has shown that other cell types have been observed to be incapable of going through MET using only cell culture techniques [36].

Within EMT, epithelial and mesenchymal phenotypes are considered endpoints in the transition. The nature of a transition lends us the argument that intermediate phenotypes exist, and correspond to partial EMT/MET. This intermediate state has been observed in wound healing and has been hypothesized to allow for establishment of equilibrium through cells capable of easily switching between phenotypes [37]. Additionally, partial EMT/MET cells have been observed during development [38], cancer [39], and fibrosis [40]. Induction of stem cell

genes, through defined culture conditions may be forcing iRC into an intermediate phenotype characteristic of MET.

MET is a well-defined mediator of pluripotency reprogramming and an established starting point for trans-differentiation. For example, it has been shown that a partial MET is useful in the direct reprogramming of mouse fibroblasts into cardiomyocytes [41]. With this in consideration, we can speculate that early stages of MET might be useful in priming cells for direct reprogramming. Further work to characterize MET would be useful in determining the state of epigenetic changes in cells undergoing reprogramming. It is still not known whether MET is responsible for complete epigenetic changes in fibroblasts, or if they still retain prior epigenetic traits. A defined culture system as described here, which induces the beginning of MET may be useful in determining epigenetic state, which is thought to have implications on future differentiations depending on the cell source trying to be created via reprogramming [42].

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Chapter 5

Thesis Conclusions

5.0 Conclusions

The work contained in the chapters of this thesis rely heavily on a body of data showing that relatively simple manipulation of a cell culture environment *in vitro* can lead to many changes in the gene expression profile of human dermal fibroblasts and the induction of stem cell genes (Oct4, Sox2, Nanog, Rex1 and Lin28) and increase the life span of human dermal fibroblasts (iRC). This culture system mediating these changes is characterized by reduced oxygen tension and the addition of exogenous FGF2. Human dermal fibroblasts (hDF) cultured under those conditions exhibit some molecular characteristics of pluripotent cells; however they do not acquire pluripotency and they do not form tumors when injected into SCID mice. One major characteristic of our *in vitro* culture system is the induction of an extended lifespan. In addition, human muscle derived fibroblasts cultured under the aforementioned conditions contribute to the regeneration of mouse skeletal muscle through functional engraftment and acquisition of a PAX7 positive satellite cell phenotype. FGF2 appears to be the critical component to this system [1, 2].

In chapter one, we tested the hypothesis that FGF2 signaling under low oxygen conditions induces changes in the expression of extracellular matrix and adhesion molecules that reflect the transition in their developmental potential – namely, a departure from producing predominantly collagen-based environment. A large number of molecular cues occur through the extracellular niche, therefore we hypothesized that the role of the extracellular environment on stem cell behavior *in vitro* is critically important for maintenance of pluripotency. We have found an overall deregulated extracellular matrix and integrin profile in human dermal fibroblasts grown in low oxygen with FGF2 supplementation (iRC). In this work we defined the overall changes in the composition ECM and in the integrin profile in human dermal fibroblasts. An ECM/AM microarray was used as an initial screen and out of 96 potential targets 25 were determined to be present in hDF and iRC cells.

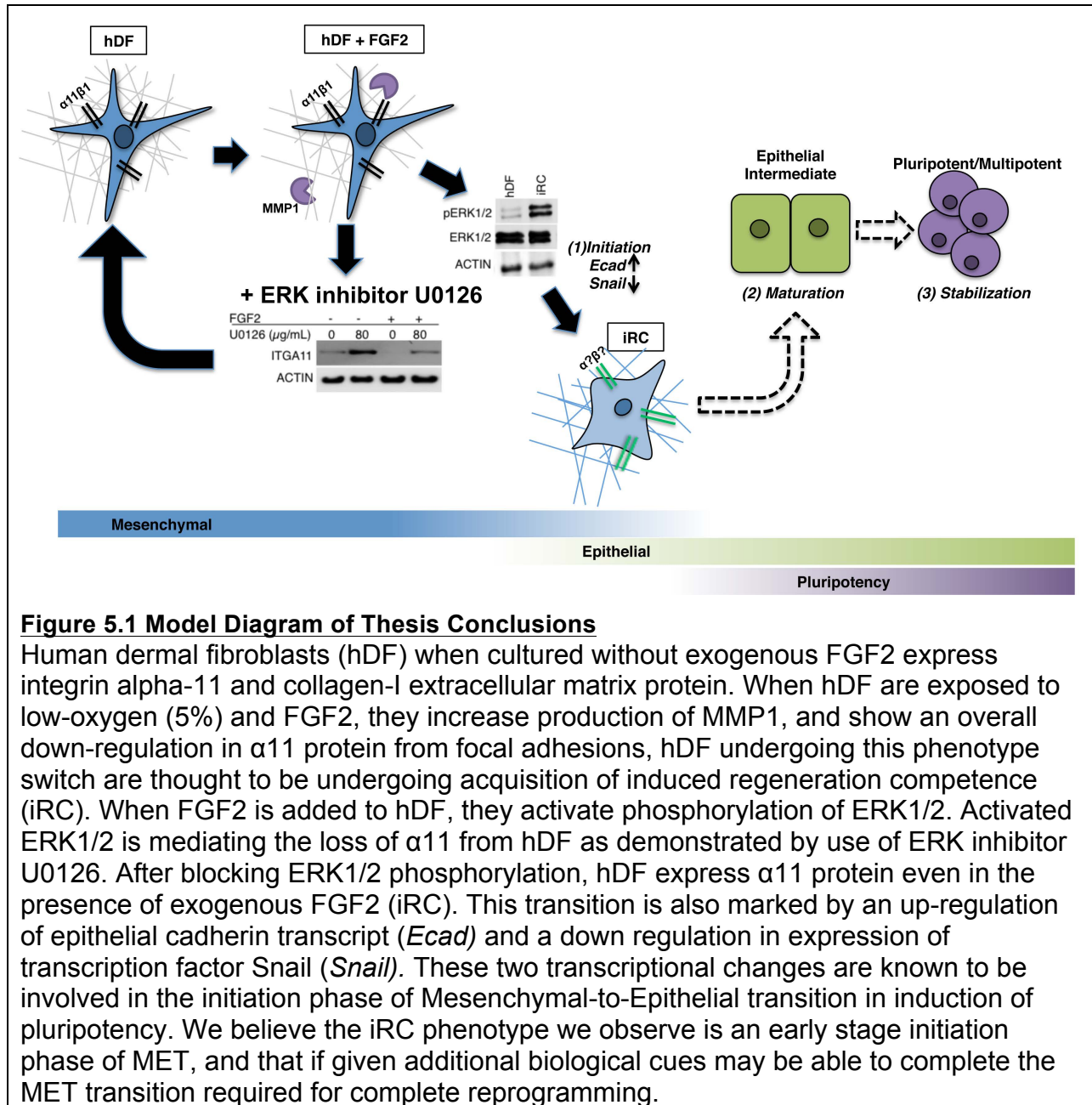
Out of those 25 targets, 15 were highly deregulated and were subject to further analysis by qRT-PCR. From the 15 targets chosen, we were able to characterize iRC by a change in niche dynamics defined by the most highly deregulated targets belonging to a similar ontology. A 17-fold down-regulation in *Itga11* (collagen-I attachment) transcript and a 5-fold *MMP1* (collagenase type-I) up-regulation led us to believe the iRC cells were remodeling the niche to remove collagen-I and subsequently, adhere to non-collagen-I substrate. By western blot, we showed that COL1 was lost from iRC cultures, as well as integrin alpha-11. Additional qRT-PCR analysis for alternate integrin alpha and beta subunits led us by subtractive analysis to determine that iRC cultures were likely adherent to RGD and laminin substrates, which were indicative of a niche with basement membrane components. A fibroblast phenotype which no longer synthesizes and anchors to collagen-I characterizes a fibroblast phenotype that is able to participate in wound healing with a less fibrotic response.

In chapter two we tested the hypothesis that low oxygen-mediated FGF2 signaling is suppressing transcriptional activity of integrin α 11 either directly or indirectly. In this chapter we investigated the mechanism of a collagen-I binding integrin α 11 (ITGA11) down-regulation in response to low oxygen-mediated FGF2 activity. We described a significant down-regulation of ITGA11 and showed that its loss requires FGF2 induced ERK1/2 activity. Moreover in the presence of FGF2, ITGA11 expression could not be rescued by TGF- β 1, a potent activator of ITGA11. This indicated that ERK1/2 mediated FGF2 down-regulation of α 11 by suppressing TGF- β 1 activity. We also showed that FGF2 is capable of overriding TGF- β 1 mediated alpha-smooth muscle actin expression, a marker of pro-fibrotic activated fibroblasts.

In chapter three we tested the hypothesis that the transition between a fibroblast and an induced regeneration competent cell proceeds through a mesenchymal-to-epithelial transition (MET), and that this transition is a consequence of down-regulation of α 11.

In chapter three we characterize the presence of focal adhesions and focal adhesion kinase activity in iRC's, and identify activation of expression of initiation stage MET genes.

These observations indicate that the activation of MET initiation stages may require a shift in extracellular matrix and adhesion molecules. We attempted to confirm a regulatory role for $\alpha 11$ in this transition, but could not achieve over-expression of $\alpha 11$. Similarly, the knock-down studies of $\alpha 11$ through transient transfection remain inconclusive. To facilitate MET, we aggregated and subsequently culture iRC in 3D aggregates. We show by Western blot and immunostaining that aggregates do not express epithelial cadherin, which is a defining marker for epithelial phenotype. The completion of MET with acquisition of an epithelial phenotype is necessary for induction of pluripotency, however further understanding the mechanism of a partial transition may lead to development of cell phenotypes for therapy without the associated risk of tumor formation.



This work has immediate potential applications in fibrosis and wound healing. We know that after injury, fibroblasts replace the wound with granulation tissue, composed of fibronectin and collagen-I and the wound is re-vascularized. In addition, fibroblasts acquire an activated, highly contractile phenotype to close the wound with deposition of collagen-I dense scar tissue. Subsequent to wound closure, remodeling events usually occur, which should revert collagen-I

dense scar tissue into functional tissue architecture [3]. In extreme cases when the routine healing process is compromised due to severe trauma, or upon deregulation normal fibrotic/healing response; irreversible fibrosis occurs, resulting in deposition of excessive fibrous connective tissue and dense scar formation. Additionally, pathological fibrosis can have implications beyond scar formation. Fibrotic pathologies compromise the function of affected organs and tissues. Irreversible fibrosis is frequently responsible for death from end-stage liver, kidney, heart diseases, idiopathic pulmonary fibrosis [4] and unresolved fibrotic conditions are features of many autoimmune diseases [5]. TGF β -1 mediates these fibrotic responses [6] through activation of ECM genes [7] and suppression of regulators of ECM degradation [8]. In fibrotic conditions, activated fibroblasts deposit large amounts of collagen-I by TGF- β 1 [9]. A feature that is common to the majority of fibrotic conditions regardless of initiating events is the activation of fibroblasts to a myofibroblastic phenotype that in many diseases are thought to be largely responsible for the deposition of extracellular matrix ultimately resulting in fibrosis. Currently, chronic fibrosis is irreversible [10]. Therapies to reverse, stop or make any improvement to an existing fibrotic tissue, or even to reverse existing tissue fibrosis are scarce. The presentation of fibrotic conditions in many organs are pathologically similar, therefore using a common tissue fibrosis pathway could yield therapeutic targets for other presentations.

Fibrosis is not just limited to wound healing, it is defined as a scarring process involving excessive deposition of extracellular matrix (ECM) and leading to impairment of organ function. Progression and worsening of chronic diseases of the liver, kidney [11], heart [12], and lung [13] have been associated with fibrosis. Fibrosis also plays a role in the skin pathologies such as scleroderma [14], inflammatory bowel disease [15] and bone marrow myelodysplastic syndromes [16].

Additionally, the stroma of solid tumors can be considered fibrotic tissue [10]. It has been recently extensively shown that wound-healing, fibrosis and cancer share common biological processes [10], and that the tumor niche resembles a site of chronic wound healing [17]. Of

many similarities between wound healing and tumor progression, the mutual presence of myofibroblastic cells has emerged as a common hallmark [18].

Integrin- α 11 has been described as contributing factor to fibrotic tumor stroma [19]. In many cases, fibrotic tumors are chemotherapy resistant, one reason being that they are highly dense, and impermeable cytotoxic chemotherapy [20]. Cancer-associated fibroblasts contribute heavily to all stages of cancer, and are an emerging target for therapy due to their participation in fibrous tumor stroma [9]. α 11 contributes to this dense environment through high levels of binding to collagen-I [19]. Mitigation of α 11 binding through a pathway independent of TGF β -1 could provide therapeutic targets for dense fibrotic tumor stroma.

The “pro-regenerative” phenotype we observe with iRC cells is capable of triggering a non-fibrotic response in dermal fibroblasts, independent of TGF β 1 mediated fibrotic response. As both TGF β 1 and FGF2 are present in the wound site and in tumors, it is likely that we are describing a physiological dynamic relationship which we hypothesize can be manipulated to address a variety of applications.

In summary, this human fibroblast conversion system allows for a cell-fate conversion from an abundant cell source to a more ‘developmentally’ plastic cell type. This phenotype has the potential for therapeutic applications in regenerative medicine, and moreover the ability to overcome the limitation of reaching a number of cells which could be useful for therapy. Identification of molecular mechanisms controlling this phenotype conversion has the potential to transition this system to other cell sources, which would broaden the range of therapeutic possibilities for autologous cell therapy.

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Chapter 6

Future Directions

6.0 Immediate Future Directions

This thesis describes an overall shift in extracellular matrix and adhesion molecules gene expression in human dermal fibroblasts undergoing the initiation phase of Mesenchymal to Epithelial Transition during conversion to induced regeneration competent cell phenotype. This transition is marked by a loss of $\alpha 11$ and its binding partner Collagen-I through a mechanism that is mediated by ERK1/2 activation and suppression of TGF- β 1-mediated regulation of $\alpha 11$. Additionally, a shift in the transcript levels of other adhesion molecules demonstrate that iRC cells are most likely transitioning to attachment to a laminin and fibronectin-based matrix, suggesting that they may be producing a more “pro-regenerative matrix”. We hypothesized that the shift in the cells ECM production and interaction with ECM serves as a feedback loop during the phenotype shift, as contact with extracellular matrix and its involvement in the regulation of potency in adult stem cells within the niche has been demonstrated extensively. We anticipate that this “pro-regenerative” shift can be exploited in wound healing biology and fibrosis research, as it triggers a non-fibrotic response in dermal fibroblasts, overriding TGF β 1-mediated fibrotic response since both TGF β 1 and FGF2 are present at the wound site. Manipulation of the dynamic relationship between the two could be used to reduce scar deposition.

Immediate directions for follow-up studies include (1) determining the FGF2 responsive elements through promoter analysis, (2) elucidating a role of FAK in the regulation of $\alpha 11$.

Determine FGF2 responsive elements through promoter analysis

In order to determine factors that may be regulating Itga11 transcription, the promoter regions of the 15 most highly deregulated genes from the ECM/AM array were compared (data not shown). The Transcription Factor Matrix Explorer (TFM-Explorer) [1] was used to analyze regulatory regions in eukaryotic genomes and assess conservation of spatial arrangements of regulatory elements. The algorithm works through two steps, 1) by scanning the sequences for all potential transcription factor-binding sites. For this comparison we used both the TRANSFAC

and JASPAR motifs databases, and 2) by extracting significant clusters and calculating a p-value. When the 15 highly deregulated genes were compared for common transcription factor elements, we determined that they all have SP1 transcription factor in common as a regulatory element.

For future work, it would be important to investigate the potential role of Sp1 in the regulation of integrin-alpha 11, and additionally investigate the potential for HLTF-1 in this process. Sp1 is able to activate transcription through association with a co-activator associated with TATA-box binding transcription factors. Sp1 is implicated in recruitment of TATA-binding protein and subsequent facilitation of the transcription initiation complex to the start site [2] even in cases of genes lacking a TATA-box [3]. When Sp1 was blocked by anti-sense Sp1 fibroblasts, they showed reduced expression of extracellular matrix associated genes [4], therefore it is likely that an interference with Sp1 binding to integrin alpha -11 promoter may be involved in regulation of its expression. Additionally, α 11 transcription has been shown to be mediated by TGF- β 1 in a Smad2/3 and Sp1 dependent manner [5, 6].

In order to further elucidate what the regulatory mechanism between ERK1/2 and integrin-alpha 11 might be, we mapped common regulatory elements between α 11 and α -sma as they both have been implicated in fibrosis and are regulated by Sp1. When they were compared (data not shown), they showed a common regulatory element, Helicase-like transcription factor (HLTF1). HLTF-1 has been shown to interact with SP1 and SP3 independently of DNA; the interaction with these transcriptional factors may be required for basal transcription of target genes [7].

Another potential regulatory mechanism for the down-regulation of integrin alpha-11 may be through activation of Vitamin D3 (VD3). A recent study looking at RUNX and VD3 in combination showed that integrin alpha-11 expression could be up-regulated by RUNX, but when VD3 was introduced in the presence of RUNX, integrin-alpha 11 transcript was decreased

[8]. Smad proteins have been shown to cross talk with several other transcription factors, and regulatory elements, such as vitamin D3 (VD3), TFE3 and Sp1 to activate transcription [9].

This would be a pathway of interest to more fully understand this process, as VD3 has been shown to be activated by MAPK signaling and additionally, its activation has also been shown to increase epithelial cadherin expression [10].

Elucidate role for FAK in FGF2 mediated down-regulation of Integrin alpha-11

It has been shown that FAK is necessary for the FGF2 mediated down-regulation of α -sma, however FAK does not contribute to a TGF β -dependent a myofibroblast phenotype [11, 12]. In the same study, FGF2 mediated signaling through ERK was shown to be dependent on FAK, however the regulation of this process is unknown [11]. It is thought that the relationship between FAK and FGF2 mediates the reversal of fibrotic phenotype and could be a a potential target for treatment of fibrotic pathology [13].

With the hypothesis that FGF2 is mediates α 11 expression in a similar mechanism to its regulation of α -sma, the role for FAK in this process may provide insight as to how FGF2 signaling regulates integrin-alpha 11 expression in the presence of TGF- β 1.

For future work, it would be of interest to investigate the pathway by which FGF2 activates FAK to induce an anti-fibrotic response in the presence of TGF- β 1. If FAK signaling is directly involved in the FGF2 mediated down-regulation of fibrotic related pathways, such as the expression of α -sma, it may also be playing a role in the FGF2-mediated down-regulation of integrin alpha-11.

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Chapter 7

Materials and Methods

7.1 Chapter 2

Cell culture

Primary adult human dermal fibroblasts (CRL-2352) were obtained from American Tissue Culture Collection (ATCC; Manassas, VA). Cells were cultured in DMEM:Ham's F12 (v:v), supplemented with 10% Fetal Clone III (Hyclone, USA), 4 mM L-glutamine at 37°C, 5% CO₂, and 5% O₂. Cells were passaged at 80% confluence using standard procedures. When used, human recombinant FGF2 (Peperotech, Rockyhill, New Jersey) was supplemented at 4 ng/mL. Human embryonic stem cells - hESCs (H9, WiCell, Madison, WI) were cultured on primary mouse embryonic fibroblasts (MEFs) derived from E13-E14 mice, and used up to passage 7. Mitomycin-C inactivated MEFs were plated at a density of 2.25×10^4 cells/cm² on tissue culture plates coated with 1% gelatin solution (MP Biologicals, Solon, OH). hESCs were plated onto inactivated MEFs and cultured in Knockout DMEM (Life Technologies, Carlsbad CA), supplemented with 15% Knockout serum (Life Technologies, Carlsbad CA), 2.0 mM Glutamax (Life Technologies, Carlsbad CA), 50 mM 2-β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 1x MEM non-essential amino acids (Mediatech, Manassas, VA) and 4 ng/mL FGF2 (Peprtech, Rocky Hill, NJ) in 5% oxygen. Medium was changed daily except for the first day after plating to allow hESCs to attach. Cells were passaged every five days. hESC colonies were dissociated using enzyme-free Cell Dissociation Buffer (Life Technologies, Carlsbad CA) and split 1:12 onto new MEFs feeder layer prepared as described previously.

ECM/AM Array:

A human pathway-focused oligo hybridization microarray for ECM and adhesion molecule (AM) targets (SA Bioscience Corp., Frederick, MD) was used to determine screen for change in transcript expression between hDF and iRC. The array contained

96 ECM/AM targets. RNA samples from both hDF and iRC was isolated as described for RT-PCR. RNA samples were labeled and amplified. cRNA was applied to each array and hybridized overnight at 60°C. Positive cRNA binding was detected by incubation with a chemiluminescent substrate, CDP-Star (Super Array). Images for the array membranes were acquired using a Gel Doc XR™ System (Bio-Rad, Hercules, CA). The images were exported at the highest resolution, and analyzed using GEArray Expression Analysis Suite Software (GEArray).

RT-PCR

RNA from cells was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol and the concentration determined with Nanodrop 2000 using A260/A280 ratio between 1.9 and 2.0 (Thermo Scientific, Waltham MA). Isolated RNA was incubated with DNase to remove any contamination with genomic DNA following the manufacturer's protocol (Agilent Technologies, Santa Clara, CA). One microgram of RNA was reverse transcribed using qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD). RT-PCR was performed using GoTaq® green master mix (Promega, Madison, WI). All RT-PCR reactions were set as follows: 20 µL reaction volume, 50 ng of cDNA template, 200 nM forward and reverse primers (Table 1). PCR was run with an initial denaturation at 95°C for 7 minutes followed by 28 cycles of the following sequence: denaturation at 95°C for 30 seconds, annealing for 30 seconds (primer-specific temperature), extension at 72°C for 20 seconds followed by a clean-up extension at 72°C for 7 minutes. RT-PCR products were resolved on 1% agarose gels containing 0.5µg/ml ethidium bromide in TAE buffer. Images were obtained on a BioRad Gel Doc™ XR system (BioRad, Hercules, CA). No template and no reverse transcriptase reactions were used as controls.

qRT-PCR

For qRT-PCR, RNA was extracted using the protocol described in RT-PCR methods. SYBR® Green Real Time PCR Master Mix (Life Technologies, Carlsbad, CA) was used as an indicator dye, with ROX as a passive dye. For each 20µL reaction, 40ng of template cDNA was used. Controls run in parallel were no-template and no-reverse transcriptase. All qPCR reactions were performed in experimental triplicates and subsequent values were combined to calculate average cycle threshold (Ct). Signal was measured using the 7500 Real Time PCR system (Applied Biosystems, Bedford, MA). Expression profiles for transcripts were calculated by the delta delta-Ct method [1] and normalization to delta-CT of actin. Expression profiles are represented as fold change in expression using the delta-delta CT method. Fold change in expression less than one is expressed as the negative inverse to account for directionality. In order to quantify the amount of miRNA29a, total small RNA was isolated from cells using NucleoSpin® miRNA kit following manufacturer instructions (Macherey-Nagel, Duren, Germany). Total miRNA quantification was performed using qScript microRNA Quantification System (Quanta Biosciences, Gaithersburg, MD). miRNA cDNA was synthesized from 1 µg of RNA using qScript microRNA cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). qPCR reactions were performed on a 7500 Real Time PCR system (Applied Biosystems, Bedford, MA). Cycling was performed as follows: 40 cycles at 95°C for 5 seconds and at 60° C for 30 seconds. PerfectCTa microRNA Assay qPCR primers were used for miRNA29a, miRNA29b, and RNU6 miRNA was used as a positive control for normalization. The specificity of primers was monitored with heat dissociation at the end of the run. All expression levels were calculated using the delta delta-Ct method [1].

Western blotting

Total protein was isolated from cells using cold lysis buffer (40 mM Tris-HCl, 250 mM NaCl, 8% glycerol, 0.0125% Brij-35, 0.01% Triton X-100, 0.005% Tween-20) supplemented with complete protease inhibitor cocktail (Roche, Basal, CH). Cells were lysed on ice using a Missonix XL-2000 ultrasonic cell disruptor for 3 x 10 pulses at power 2. Total cell lysate was mixed with 5X Laemmli sample buffer. Samples were boiled for 5 minutes and separated on 4-20% SDS-PAGE gels (Bio-Rad). For Western blot analysis, protein was transferred to PVDF membrane (Millipore, Billerica, MA) using a semi-dry transfer apparatus (GE Healthcare, Fairfield, CT). Membrane was blocked in TBS-T buffer (25 mM Tris-HCl pH7.5, 130 mM NaCl, 0.1% Tween-20) with 5% fat-free dry milk for 30 minutes. The membrane was incubated overnight with 1% fat-free dry milk at 4°C with primary antibodies: anti-ITGA11 (MAB4235, 1:500, RD Systems), anti-TGFβ1 (9758, 1:500, Abcam), anti-COLIA1 (sc-8783, 1:500, Santa Cruz), anti-Fibronectin (sc-9068, 1:500, Santa Cruz), anti-p44/42 MAPK (Erk1/2) (4695, 1:1000, Cell Signaling Technologies), anti- Phospho-p44/42 MAPK (Erk1/2) (4370, 1:1000, Cell Signaling Technologies), anti-FAK (13009, 1:500, Cell Signaling Technologies), anti-Phospho-FAK (Tyr397) (8556, 1:500, Cell Signaling Technologies), anti-Phospho-FAK (Tyr576/577) (3281, 1:500, Cell Signaling Technologies), anti-Phospho-FAK (Tyr925) (3284, 1:500, Cell Signaling Technologies). After washing the membrane 3 x 10 minutes in TBS-T, the membrane was incubated for 2 hours at room temperature in appropriate HRP-conjugated secondary antibodies (BioRad, Hercules, CA) . The bands were visualized using a Gel Doc XR™ System (Bio-Rad).

7.2 Chapter 3

Cell culture

Primary adult human dermal fibroblasts (CRL-2352) were cultured as indicated in previously in chapter 2 materials and methods. When indicated, human dermal fibroblasts were incubated with 10ug/mL alpha-amanatin to block new transcription. When indicated, dermal fibroblasts were stimulated with TGF β 1 at concentrations ranging from 0.05 to 50 ng/mL. Cultures were treated with TGF β 1 for seven days in parallel with FGF2 supplemented cultures. For ERK inhibitor studies, cells were seeded at a density of 5000 cells / cm² in order to have sufficient material for harvest. Cells were serum starved and, when noted, treated with ERK1/2 inhibitor (U0126) (Cell Signaling Technologies, MA) at 80ug/mL for 2 hours. After incubation, medium was replenished with complete medium for each variable, which was supplemented with FGF2, U0126 or a combination. Cells were allowed to grow for 72 hours and then harvested for analysis. When assaying for ERK1/2 activation, cells were treated as above and harvested 1 hour after being returned to complete medium in order to verify that ERK1/2 phosphorylation had been blocked sufficiently.

RT-PCR and RT-qPCR analysis

All RNA, RT-PCR and RT-qPCR were performed as described previously in chapter 2 materials and methods. In order to quantify the amount of miRNA29a, total small RNA was isolated from cells using NucleoSpin® miRNA kit following manufacturer instructions (Macherey-Nagel, Duren, Germany). Total miRNA quantification was performed using qScript microRNA Quantification System (Quanta Biosciences, Gaithersburg, MD). miRNA cDNA was synthesized from 1 μ g of RNA using qScript microRNA cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). qPCR reactions

were performed on a 7500 Real Time PCR system (Applied Biosystems, Bedford, MA). Cycling was performed as follows: 40 cycles at 95°C for 5 seconds and at 60° C for 30 seconds. PerfectCTa microRNA Assay qPCR primers were used for miRNA29a, miRNA29b, and RNU6 miRNA was used as a positive control for normalization. The specificity of primers was monitored with heat dissociation at the end of the run. All expression levels were calculated using the delta delta-Ct method [1].

Immunocytochemistry:

Fibroblasts were grown on glass coverslips for immunostaining. Cells were fixed with ice-cold methanol or 2% methanol-free formaldehyde. Samples were permeabilized with 1M HCl or 0.1 % TritonX-100, respectively. Samples were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween-20 (TBS-T, 25 mM Tris-HCl pH 7.5, 130 mM NaCl, 0.1 % Tween-20). Primary antibodies were diluted in TBS-T supplemented with 1 % BSA. Cells were labeled for 2 hours at room temperature. Plates were washed three times with TBS-T and secondary antibodies conjugated to either AlexaFluo-568, or AlexaFlour-488 (Invitrogen, Carlsbad CA) diluted in TBS-T with 1 % BSA for 1 hour. Coverslips were mounted using Prolong Gold® (Invitrogen, Carlsbad CA). Incubation with secondary antibody alone and rabbit IgG were used as negative controls. Images were acquired using confocal microscopy at identical settings.

DNA methylation analysis

The following assay was developed as a modification of [2]. Genomic DNA was purified using Quick-gDNA™ MiniPrep (Zymo Research, Irvine, CA). DNA was quantified with Nanodrop 2000 using an A260/A280 ratio (Thermo Scientific, Waltham, MA). To determine the genomic locations of CpG islands within integrin alpha-11 promoter, the Transcriptional Regulatory Element Database was used, and CpG islands were mapped using MethPrimer software. Tiling primers were designed within the two

CpG islands mapped within the promoter region of integrin alpha-11 using NCBI Primer Blast. Control genomic DNA (New England Biolabs, Ipswich MA) was used in addition to DNA from experimental treatments. One hundred nanograms of total DNA was subjected to either a methylation-sensitive restriction enzyme (MSRE), methylation-dependent restriction enzyme (MDRE) or a sham digest. Enzymes used were HhaI (MSRE) and McrBC (MDRE) (New England Biolabs, Ipswich MA). After digestion, DNA samples were cleaned over NucleoSpin® gDNA Clean-up XS (Macherey-Nagel, Duren, Germany). PCR for each primer pair was run on each digestion, genomic control DNA and a sham digestion. PCR was performed using GoTaq® green master mix (Promega, Madison WI). All PCR reactions were set as follows: 20uL reaction volume, 50 ng of DNA template, 200 nM forward and reverse primers each. PCR program was run with an initial denaturation at 95°C for 7 minutes followed by 28 cycles of the following sequence: denaturation at 95°C for 30 seconds, annealing 30 seconds (primer-specific temperature), extension at 72°C for 20 seconds, and a clean-up extension at 72°C for 7 minutes. A no template control was run in parallel. PCR products were resolved on 2% agarose gels containing 0.5 µg/ml ethidium bromide in TAE buffer. Images were acquired with a BioRad Gel Doc™ XR system (BioRad, Hercules CA).

Western blotting

Western blotting was performed as described previously in chapter 2. Where indicated, membranes were probed with primary antibodies: anti-ITGA11 (MAB4235, 1:500, RD Systems), anti-TGFβ1 (9758, 1:500, Abcam), anti-p44/42 MAPK (Erk1/2) (4695, 1:1000, Cell Signaling Technologies), anti- Phospho-p44/42 MAPK (Erk1/2) (4370, 1:1000, Cell Signaling Technologies), anti-actin (A2066, 1:10,000, Sigma).

7.3 Chapter 4

Cell culture

Primary adult human dermal fibroblasts (CRL-2352) were cultured as indicated in previously in chapter 2 materials and methods. Where indicated iRC cells were subjected to forced aggregation to try to achieve an epithelial phenotype. iRC cells were cultured in 2D for seven days, and subsequently transferred to low attachment culture dishes (Corning) where they were cultured for an additional three days. Cells were then transferred to hanging drops, and aggregates were formed in 20uL at 4000 cells per aggregate. Cells were then cultured statically for 2 days for aggregation to take place, and were subsequently transferred to low attachment plates for an additional 14 days. Aggregates were assayed at day 7 and day 14 for epithelial markers (Figure 7.1).

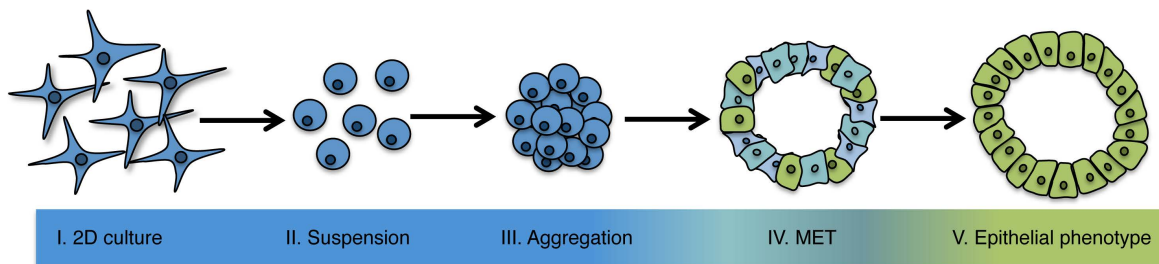


Figure 7.1 A schematic showing how cells were transitioned from 2D to 3D culture Methods based on Corwin et al., 2007.

ITGA11 Cloning

Itga11 cDNA (clone ID HsCD00295137) construct was purchased from Harvard Plasmid Repository. cDNA clone was in a pENTR223.1 Gateway cloning vector (Figure 4.6a). Primers were designed to amplify itga11 cDNA for subcloning into a pcDNA3.1-CT-GFP-TOPO[®] vector (Invitrogen) (Figure 4.2b). In order to assure expression of GFP tagged $\alpha 11$ it was necessary to introduce a point mutation to interrupt the stop codon.

Due to high GC content, and amplicon length LongAmp *Taq* (New England Biolabs) was used. Ligation into pcDNA3.1-CT-GFP-TOPO requires 3' deoxyribose adinine (dA) overhangs which are sometimes lost with high fidelity enzymes, addition of dA overhangs was done using *Taq* DNA polymerase (New England Biolabs) and dATP incubated at 72°C for 20 minutes.

Ligations were transformed into *E.coli* One Shot® TOP10 Competent cells. Positive transformants were selected with 100µg/mL ampicillin. Colonies were grown for 16-18 hours, selected and grown in LB broth containing 100µg/mL ampicillin. Plasmid was isolated using a NucleoSpin® Plasmid isolation kit. pcDNA3.1-Itgα11-GFP was sequenced and insert orientation verified using restriction digestion at *SmaI* and *BglII* sites (Figure 4.6c). Colonies selected for proper insert orientation were clones 6 and 12 (Figure 4.6c) and subjected to sequencing using M13 forward and M13 reverse sequencing primers. Sequencing results were analyzed using the basic local alignment tool (BLAST) on the on the national center for biotechnology information (NCBI) website, against the human cDNA library to determine sequence identity and verify the accuracy of stop codon PCR-based mutagenesis.

Transfection

Expression verification was performed in HeLa and CHO-K1 cells with transient (24-48 hour) and transfections using lipofectamine 2000 (Invitrogen) with endotoxin free plasmid DNA. RT-PCR, Western blot and live imaging techniques were used to verify expression and localization Itgα11-GFP. Cells were grown in 10cm tissue culture dishes to 70% confluence prior to transfection with pcDNA3.1-Itgα11-GFP isoforms. pcDNA3.1-Itgα11-GFP were transfected in cells using Lipofectamine® 2000 (Life technologies). Cells were incubated with lipofectamine/plasmid DNA in 50/50 DMEM (Hyclone), Ham's F12 (Hyclone), 10% FBS (Hyclone) for 18 hours then fresh media was

added to the cells. Cells were harvested and subjected to Western blot analysis and chromatin immunoprecipitation 48 hours post transfection. Adult human dermal fibroblasts CRL2352 (ATCC) were also grown in 10cm tissue culture dishes at 80% confluency prior to transfection with individual FGF2 isoforms. Cells were incubated in 50/50 DMEM (Hyclone), Ham's F12 (Hyclone), 10% Fetal Clone III (Hyclone) with lipofectamine/plasmid DNA using the same expression vectors described above. Media were replaced with fresh growth media 8 hours post transfection. Western blot, RT-PCR and live imaging were used to verify expression and localization Itg α 11-GFP.

For human dermal fibroblast transfection, cells were electroporated with endotoxin free plasmid DNA. Approximately 1×10^6 cells were electroporated in 100 μ l of electroporation buffer with three pulses with 10 seconds rest between each pulse at 112mV for 1 msec/pulse (BTX). Electroporated cells were resuspended in complete medium. Western blot, RT-PCR and live imaging was used to verify the expression and localization of Itg α 11-GFP.

RT-PCR and RT-qPCR analysis

All RNA, RT-PCR and RT-qPCR were performed as previously described in chapter 2 materials and methods.

Immunohistochemistry

Cell aggregates were washed in PBS and fixed in 2% paraformaldehyde for 2 hours, rinsed with PBS and processed through alcohols, cleared in xylenes, embedded in paraffin wax, sectioned into 5 μ m slices, and mounted onto charged microscope slides. Paraffin wax was removed from the tissue sections by 3 incubations in Xylene, followed by hydration in graded alcohols and a 5-minute wash in diH₂O. Antigen retrieval was conducted in a pressure cooker for 20 minutes in a citrate-based antigen retrieval solution at a 1:100 dilution in diH₂O (Vector Labs). Incubating tissue sections in 3%

hydrogen peroxide for 15 minutes quenched endogenous peroxidase activity. Non-specific binding sites were blocked using 2.5% serum of the species producing the secondary antibody diluted in PBS for 15 minutes. Staining was conducted using primary antibodies specific to E-Cadherin (1:200, Santa Cruz). Species-specific highly sensitive polymerized reporter enzymatic staining system (ImmPRESS Kits, Vector Labs) was used to conjugate horseradish peroxidase micropolymers to affinity-purified secondary antibodies with detection achieved by ImmPACT DAB peroxidase substrate (Vector Labs). Nuclei were counterstained with Harris Hematoxylin, dehydrated through graded alcohols, cleared in Xylenes, permanently mounted and imaged using a Zeiss Axiovert inverted fluorescent microscope.

Western blotting

Western blotting was performed as described previously in chapter 3. Where indicated, membranes were probed with primary antibodies: anti-ITGA11 (MAB4235, 1:500, RD Systems), anti-GFP (JL-8, 1:1000, Clontech,) anti-FAK (13009, 1:500, Cell Signaling Technologies), anti-Phospho-FAK (Tyr397) (8556, 1:500, Cell Signaling Technologies), anti-Phospho-FAK (Tyr576/577) (3281, 1:500, Cell Signaling Technologies), anti-Phospho-FAK (Tyr925) (3284, 1:500, Cell Signaling Technologies), anti-ECAD (sc-7870, 1:1000, Santa Cruz Biotechnologies), anti-ITGA11 (MAB4235, 1:500, RD Systems), anti-actin (A2066, 1:10,000, Sigma).

7.5 References

1. Schmittgen TD, Livak KJ: **Analyzing real-time PCR data by the comparative CT method.** *Nature protocols* 2008, **3**(6):1101-1108.
2. Oakes CC, La Salle S, Robaire B, Trasler JM: **Evaluation of a Quantitative DNA Methylation Analysis Technique Using Methylation-Sensitive/Dependent Restriction Enzymes and Real-Time PCR.** *Epigenetics* 2006,**1**(3):146-152.

Appendix 1: Genes Identified from Array

GeneBank	Symbol	Description	GO Term
NM_002026	FN1	Fibronectin 1	Cell adhesion;Extracellular;Oxidoreductase activity;Collagen binding;Extracellular matrix structural constituent;Heparin binding;Acute-phase response;Cell migration;Metabolism;Response to wounding;
NM_002211	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	Integral to membrane;Protein binding;Development;Receptor activity;Homophilic cell adhesion;Cell-matrix adhesion;Integrin-mediated signaling pathway;Integrin complex;Protein heterodimerization activity;Cellular defense response;
NM_002205	ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	Integral to membrane;Protein binding;Receptor activity;Cell-matrix adhesion;Integrin-mediated signaling pathway;Integrin complex;
NM_002290	LAMA4	Laminin, alpha 4	Receptor binding;Protein binding;Extracellular matrix structural constituent;Regulation of cell adhesion;Regulation of cell migration;Regulation of embryonic development;Laminin-1;
NM_003118	SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	Calcium ion binding;Collagen binding;Ossification;Basement membrane;
NM_000358	TGFBI	Transforming growth factor, beta-induced, 68kDa	Cell adhesion;Cell proliferation;Protein binding;Negative regulation of cell adhesion;Extracellular matrix;Extracellular space;Integrin binding;Visual perception;Extracellular matrix (sensu Metazoa);
NM_001846	COL4A2	Collagen, type IV, alpha 2	Cytoplasm;Extracellular matrix structural constituent;Phosphate transport;Collagen;Extracellular matrix organization and biogenesis;Collagen type IV;
NM_001850	COL8A1	Collagen, type VIII, alpha 1	Cell adhesion;Protein binding;Cytoplasm;Extracellular matrix structural constituent;Phosphate transport;Extracellular matrix (sensu Metazoa);Collagen type VIII;
NM_004385	VCAN	Versican	Calcium ion binding;Development;Hyaluronic acid binding;Cell recognition;Sugar binding;Extracellular matrix (sensu Metazoa);
NM_001004439	ITGA11	Integrin, alpha 11	Integral to membrane;Receptor activity;Collagen binding;Cell-matrix adhesion;Muscle development;Integrin-mediated signaling pathway;Integrin complex;Magnesium ion binding;
NM_002204	ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	Integral to membrane;Protein binding;Receptor activity;Cell-matrix adhesion;Integrin-mediated signaling pathway;Integrin complex;
NM_002421	MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	Hydrolase activity;Calcium ion binding;Zinc ion binding;Interstitial collagenase activity;Collagen catabolism;Extracellular matrix;Extracellular space;Extracellular matrix (sensu Metazoa);
NM_004530	MMP2	Matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	Hydrolase activity;Calcium ion binding;Zinc ion binding;Collagen catabolism;Extracellular matrix;Extracellular space;Gelatinase A activity;Extracellular matrix (sensu Metazoa);
NM_002422	MMP3	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	Hydrolase activity;Calcium ion binding;Zinc ion binding;Collagen catabolism;Extracellular space;Stromelysin 1 activity;Extracellular matrix (sensu Metazoa);
NM_003919	SGCE	Sarcoglycan, epsilon	Calcium ion binding;Protein binding;Cell-matrix adhesion;Integral to plasma membrane;Cytoskeleton;Muscle development;Sarcoglycan complex;

Appendix 2: Primers for all PCR

Gene	Sequence (5'—3')
CavIL	GGATGTGCCTAGACCCGGCG
CavIR	GAACGGTGTAGAGATGTCCCTCCGA
CavIIL	GCAGAGCCGGTGACTACGCA
CavIIR	AGCATCGTCCTACGCTCGTACACA
CavIIIL	CGGCCACACAGCTCGGATCTC
CaIIIR	AGCTCACCTTCCACACGCCG
ILKL	AGCCCGAGTCCCGAGGATAAAGC
ILKR	CTCGGCAGGCCCCAGTGCAAG
CTNNB1L	CCAATGGCTTGGAAATGAGACTGCTG
CTNNB1R	CACCTGGAGGCAGCCCATCC
FYNL	CGTGGAAAAGACCAGTCCTGCCT
FYNR	CCCGGCTGCGTGGAAGTTGT
ILKL	GAGTCCCGAGGATAAAGCTTGGGGT
ILKR	CTCGGCAGGCCCCAGTGCAAG
FN1L	CTGGCCGAAAATACATTGTAAA
FN1R	CCACAGTCGGGTCAGGAG
ITGB1L	CGATGCCATCATGCAAGT
ITGB1R	AACAATGCCACCAAGTTTCC
ITGB5L	GGGCCTTTCTGTGAGTGC
ITGB5R	TGTAACCTGCATGGCACTTG
LAMA4L	AAGCGACCTGCAAGCAAC
LAMA4R	GCCATCAAACATCATGGAGAC
SPARCL	TTCCCTGTACACTGGCAGTTC
SPARCR	AATGCTCCATGGGGATGA
TGFB1L	CATTGGTGATGAAATCCTGGT
TGFB1R	TGACACTCACCACATTGTTTTTC
COL4A2L	GAGAAGGCGCACAACCAG
COL4A2R	CCGGCTGGCATAGTAGCA
COL8A1L	CATCTCAAGAACAAAAGACAACCTGA
COL8A1R	TTGCTGGTGCCTTCCTGT
ITGA11L	GGTCTGTAAAAGATGTGGTGGAA
ITGA11R	CTTCTGGAAAGCCTCTGAGC
ITGA3L	CCCTCACTCCTTCTTCATGG
ITGA3R	GAGCTCCCACAGCAATATCC
MMP1L	GCATATCGATGCTGCTCTTTC
MMP1R	GATAACCTGGATCCATAGATCGTT
MMP2L	CGATGATGACCGCAAGTG
MMP2R	GGTCTTGGGAGTGCTCCAG
MMP3L	CTCCAACCGTGAGGAAAATC
MMP3R	CATGGAATTTCTCTTCTCATCAA

SGCEL	CCACTGGCACATTCTTGCT
SGCER	TTCCAAAACATGAACAAAGAGG
SPARCL	GGAGGTGGTGGCGGAAAATCCC
SPARCR	GGGCCTGGCACACGCACAT
CAMK2N1L	GCCGGAGCAAGCGGGTTGTTA
CAMK2N1R	CACCAGGAGGTGCCTTGTCGG
MMP3L	ACCTGACTCGGTTCCGCCTG
MMP3R	GTCAGGGGGAGGTCCATAGAGGG
SPP1L	GGCATCACCTGTGCCATACCAGT
SPP1R	TGGAAGGGTCTGTGGGGCTAGG
FN1L	CTGGCCTGGAACCGGGAACC
FN1R	ACCAGTTGGGGAAGCTCGTCTGT
DSPL	ATCAAAGCCGACCTGCGCGA
DSPR	AGCAGCTCCTCCTCCTCGCA
TCF4L	AGCCATGGGTGGTCTGGGCT
TCF4R	ACGATGGGTCCCCACCATGA

Primer	Sequence (5'—3')	Amplicon(bp)
ITGA11CpG1L	GGAGCGGTTCTCTGGAGTTT	196
ITGA11CpG1R	TGCGGCTCATTAGAGGAAG	
ITGA11CpG2L	CTCCGGGGAAGCATCTCAA	109
ITGA11CpG2R	CTTTGCTCTGTCTTTGGCCG	
ITGA11CpG3L	CCGGGACTCTTTGGTGTGTT	197
ITGA11CpG3R	TCTTGGCGCTGATTCCCTTC	
ITGA11CpG4L	CTCCGGGGAAGCATCTCAA	109
ITGA11CpG4R	CTTTGCTCTGTCTTTGGCCG	
ITGA11CpG5L	TAATGCACTCCAGGTGTCGG	180
ITGA11CpG5R	TTCCGCAGAACAGCATCACT	