ROLE OF FIBROBLAST GROWTH FACTOR-2 IN MAINTENANCE OF MULTIPOTENCY IN HUMAN DERMAL FIBROBLASTS TREATED WITH *XENOPUS LAEVIS* EGG EXTRACT FRACTIONS

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By

Denis Kole

Approved by:

Tanja Dominko, D.V.M., Ph.D
Associate Professor, Advisor
Biology and Biotechnology
Worcester Polytechnic Institute

David S. Adams, Ph.D
Professor
Biology and Biotechnology
Worcester Polytechnic Institute

Joseph Duffy, Ph.D
Associate Professor
Department Head
Biology and Biotechnology
Worcester Polytechnic Institute

Scott Gridley, Ph.D
Vice President
Business Development
Blue Sky BioServices Inc.

Abstract

Current usage of human embryonic stem cells (hES) and induced pluripotent stem cells (iPS) in clinical therapies and personalized medicine are limited as a result of ethical, technical and medical problems that arise from isolation and generation of these cells. Isolation of hES cells faces ethical problems associated with their derivation from human pre-implantation embryos. The most controversial aspect of hES cell isolation targets the generation of autologous hES cell lines which requires the transfer of a somatic-cell nucleus from the patient to an enucleated oocyte. While already established embryonic stem cell lines from IVF embryos can be used in a similar manner, lack of genetic identity can cause therapy rejection from the host, and prevent their use in personalized medicine. Induced pluripotent stem cells on the other hand, are generated from somatic cells that have been reprogrammed in vitro to behave like stem cells. While these cells can potentially be used for personalized medicine without the risk of rejection by the host system, derivation methods prevent their therapeutic use. The most efficient method used to generate iPS cells involves usage of viral particles which can result in viral DNA being integrated in the host cell's genome and render these cells non-compliant for clinical therapies. Other methods not involving viral particles exist as well, but the reprogramming efficiency is too low and technical problems with generating large enough numbers of cells prevent these methods from being feasible approaches for clinical therapies. Direct reprogramming of a differentiated cell into a developmentally more plastic cell would offer alternatives to applications in regenerative medicine that currently depend on either embryonic stem cells (ES), adult stem cells or iPS cells.

We hypothesize that *Xenopus laevis* egg cytoplasmic extract contains critical factors needed for reprogramming that may allow for non-viral, chemically defined derivation of human induced pluripotent/multipotent cells which can be maintained by addition of exogenous FGF2.

In this thesis we investigated a new method for generation of multipotent cells through determining the ability of select fractions of *Xenopus laevis* egg extract to induce multipotency in already differentiated cells. We were able to identify select fractions from the extract that in combination with exogenously added FGF2 can reprogram and maintain the reprogrammed cells in an undifferentiated state. The findings of this work also determined that *Xenopus laevis* egg extract mRNA is required for achieving full reprogramming. The body of work presented in this thesis showed the ability of FGF2 isoforms to bind and activate select FGF receptor tyrosine kinases, act as extracellular mitogenic factors to support growth of hES cells in an undifferentiated state as well bind to nuclear DNA and affect expression of endogenous genes. Moreover, we showed that all FGF2 isoforms can induce expression of stem cell specific proteins in human dermal fibroblasts as well as extend lifespan of human dermal fibroblasts in vitro. In this work we identified HECW1, the gene coding for E3 ubiquitin ligase NEDL1, as a novel nuclear target for all FGF2 isoforms and showed that overexpression of recombinant FGF2 isoforms in human dermal fibroblasts can down regulate expression of HECW1 gene.

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Abbreviations

ANOVA: Analysis of variance

BrdU: Bromodeoxyuridine

BSA: Bovine serum albumin

BSP: Bone sialoprotein

DAG: Diaglycerol

DMEM: Dulbecco's modified eagle's medium

EC: Embryonic carcinoma

ECM: Extracellular matrix

EMSA: Electromobility shift assay

ES: Embryonic Stem

F12: Ham's F12

FACS: Fluorescence activated cell sorting

FALS: Familial amyotrophic lateral sclerosis

FBS: Fetal bovine serum

FCS: Fetal calf serum

FGF2: Fibroblast growth factor 2

FGFR: Fibroblast growth factor receptor

FPLC: Fast protein liquid chromatography

FRE: FGF2 response element

FSP: Fibroblast specific protein

GFP: Green fluorescent protein

hCG: Human chorionic gonadotropin

hESC: Human embryonic stem cells

HMW: High molecular weight

HSPG: Heparin sulfate proteoglycan

ICC: Immunocytochemistry

IL-4: Interleukin 4

IMAC: Immobilized metal affinity chromatography

iPSC: Induced pluripotent stem cells

LMW: Low molecular weight

MAPK: Mitogen activated protein kinase

MEF: Mouse embryonic feeder

NLS: Nuclear localization sequence

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PD: Population doubling

PDGF: Platelet-derived growth factor

PKC: Protein kinase C

PLCϒ: Phoshpolipase C ϒ

PVDF: Polyvinylidene difluoride

SCID: Severe combined immunodeficiency

SCNT: Somatic cell nuclear transfer

SD: Standard deviation

SDS: Sodium dodecyl sulphate

SOD-1: Superoxide dismutase-1

TAE: Tris acetate EDTA

TBST: Tris buffered saline tween

TEV: Tobacco etch virus

TGFβ: Transforming growth factor β

Introduction

Identification and characterization of human embryonic stem cells (hESC) and the ability to grow them *in vitro* marked an important milestone not only in the stem cell biology field but in clinical research and personalized medicine as well (Thomson et al., 1998). In parallel, work in reproductive cloning involving somatic cell nuclear transfer (SCNT) experiments and the success in the reproductive cloning field during the 1990s served as the foundation for therapeutic cloning work (Wilmut et al., 1997). The understanding that a series of molecular events occurring during reprogramming of the chromatin in terminally differentiated somatic cells can lead to change in gene expression profile was a foundation for extensive research efforts that in 2006 culminated with generation of murine induced pluripotent stem cells (iPS) (Takahashi and Yamanaka 2006). A year later Yamanaka and his group published on the successful generation of human iPS cells from differentiated cells (Takahashi et al., 2007). These studies showed that it was possible to reprogram a once thought terminally differentiated cell into a pluripotent cell without the need for the factors contained in mature eggs or early embryos. This was a critical achievement, as it allowed for derivation of patient- and disease-specific cell types and enabled the realization of personalized cell therapy. Today, a range of natural (embryonic and adult stem cells) and artificial (induced pluripotent stem cells) sources of stem cells and the ability to better understand and control their fate has opened the door to development of a wide range of potential stem cell-based therapies. Personalized medicine utilizing stem cell therapy is continuously growing as a promising new approach for stimulating tissue regeneration, treatment of degenerative diseases, generation of tissues and organs that can be used for transplantation, and generation of tissue models for diagnostic and drug screening purposes.

The successful implementation of personalized stem cell therapies depends on generation of clinically compliant pluripotent/multipotent cells that can be safely and ethically used for treatment of patients. Major disadvantages still limit the use of available pluripotent/multipotent cells and discovery

of new methods for generation of clinically relevant patient-specific pluripotent/multipotent cells and understanding of molecular mechanisms governing stem cell fate is of vital importance to the stem cell biology field for the advancement of stem cell therapies.

Personalized cell therapy using pluripotent and multipotent cells is becoming highly important for treatment of health conditions that so far have had little to no sustainable cures; however, stem cell therapy is not a new concept. Bone marrow derived hematopoietic stem cells have been used for transplantation during the last few decades for treatment of several hematopoietic diseases. First reports of successful cell transplantation date back to 1957 with the transplantation of bone marrow between identical twins to treat leukemia (Thomas et al., 1957). Dr. Thomas' work marked the beginning of stem cell therapy field as we know it today (Figure 1.1).

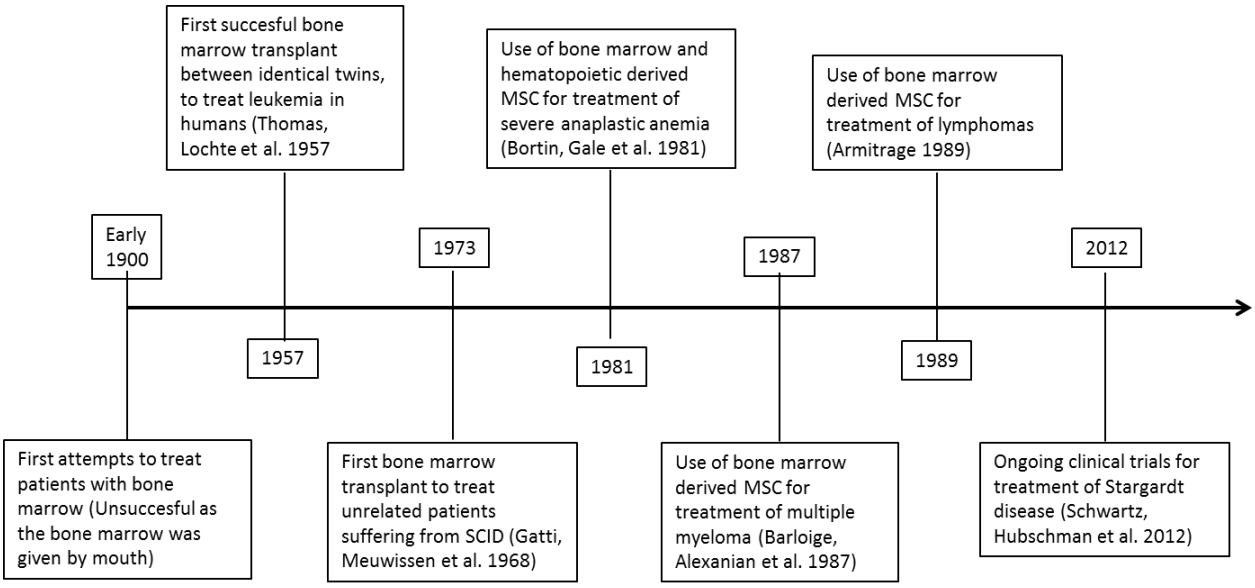

Figure 1.1 | **Abbreviated chronology of stem cell therapy field**

It wasn't until 1968 that the first successful allogeneic transplant was performed to treat a patient with severe combined immunodeficiency (SCID) (Gatti et al., 1968). Following these studies, bone marrow hematopoietic stem cell transplantation became a more common practice used for treatment of leukemias (Cassileth et al., 1998; Horowitz et al., 1990) , lymphomas (Armitage 1989;

Freedman et al., 1999; Freedman et al., 1990; Gulati et al., 1992), multiple myeloma (Attal et al., 2003; Attal et al., 1996; Barlogie et al., 1987), severe anaplastic anemia (Bortin et al., 1981; Passweg and Marsh 2010) and some solid tumors (Rodenhuis et al., 2003).

Skin

More recently, stem cells have been utilized in a few regenerative medicine applications. Reports published in 2005 described healing of an extensive skin burn after transplantation of bone marrow derived mesenchymal stem cells (MSC) (Rasulov et al., 2005). Even though the methods for treatment of extensive skin burns have proven to be lifesaving for patients, engineered skin is not structurally and functionally equivalent to normal skin. Ongoing studies are exploring iPS cells derived from different sources that can then be combined and differentiated into all the cell types of the skin allowing for a more effective regenerative response (Aasen et al., 2008).

Central nervous system

According to 2012 heart disease and stroke statistics approximately 790,000 people experience a new or recurring stroke each year (Roger et al., 2012). In the USA 4-million patients suffer consequences of ischemic stroke (Taylor et al., 1996) and stroke leads to brain cell death which results in loss of neurons and oligodendrocytes. Current treatments for acute ischemic stroke include arterial recanalization and subsequent reperfusion (Molina 2011); however neuroprotection in stroke patients is not a very successful, straight forward process and currently no good treatments exist that can restore lost cells. Stem cell therapy for treatment of stroke patients to restore neural networks and their functionality has gained interest in the recent years. Pre-clinical studies conducted with adult stem cells have shown improvement in animal models of stroke (Chen et al., 2001; Li et al., 2002). These studies showed improved motor and cognitive functions in rats with stroke. Peripheral injection of MSCs has demonstrated that these cells can cross the blood-brain barrier, reduce apoptosis and promote cellular proliferation in the damaged area (Chen et al., 2003). To date no definitive therapies exist to treat stroke

patients using stem cells, however, several studies have reported small improvements in motor neuron function in patients treated with human neuronal cells derived from pluripotent cells (Kondziolka et al., 2005; Kondziolka et al., 2000) and autologous mesenchymal stem cell transplantation (Bang et al., 2005)

Cardiovascular system

Coronary artery disease is a major health problem in the United States and the American Heart Association estimates that about every 34 seconds someone in the United States has a myocardial infarction (Thygesen et al., 2012) and coronary heart disease was responsible for 1 of every 6 deaths in the US in 2008 (Roger et al., 2012). Myocardial infarctions result in cardiomyocyte death, which impairs normal heart function and can lead to one's death depending on the severity. Current treatments for myocardial infarction include surgical intervention and pharmacological treatments to restore normal blood flow in the infarct related arteries and partially restore muscle functionality at the infarcted region (Piot et al., 2008; Stone et al., 2008). Recently, studies aimed at the use of stem cells for treatment of myocardial infarction have gained importance and animal studies have demonstrated very positive outcomes for treatment of myocardial infarctions with mesenchymal stem cells or stem cell-derived cardiovascular cells (Arminan et al., 2010; Kocher et al., 2001; Kraehenbuehl et al., 2011; Min et al., 2002; Orlic et al., 2001). 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 (Clifford et al., 2012; Martin-Rendon et al., 2008).

Vision

The World Health Organization reports that 285-million people worldwide are estimated to be visually impaired and no pharmacological or surgical treatments exist for many of the conditions that affect vision or cause blindness. Recent work in the stem cell field has provided new opportunities for the treatment of visually impaired patients. Transplantation of stem cells over the damaged cornea has been reported to stimulate, repair and restore vision (Graham-Rowe 2004) in some patients. Embryonic stem cells differentiated into photoreceptors *in vitro* and implanted into blind animals have shown good engraftment (Gonzalez-Cordero et al., 2013) and promise future success for treatments in humans. Currently, ongoing clinical trials for treatment of Stargardt disease and dry age-related macular degeneration are exploring transplantation of retinal pigmented epithelial cells (RPE) derived from hES cells (Schwartz et al., 2012).

Musculoskeletal system

Skeletal muscle has a limited ability to fully regenerate muscle fibers after large damage by injury or as a result of skeletal muscle diseases (Mann et al., 2011). Lack of effective treatments for volumetric muscle loss and skeletal muscle conditions such as sarcopenia, is one of the main reasons why patients suffering from these conditions never fully recover, but at the same time lack of effective treatments makes stem cell therapy an attractive option. Recent animal studies have reported improvements in skeletal muscle functionality of skeletal muscle conditions such as muscular dystrophy after embryonic stem cell transplantation (Darabi et al., 2008). Generation of myogenic cells from adult stem cells, hES cells or iPS cells can be used as personalized therapy for treatment of skeletal muscle diseases.

The invention of iPS cells (Takahashi and Yamanaka 2006) and the increased understanding on how to control stem cell fate *in vitro*, has made hES cells and iPS cells an attractive approach for use in personalized regenerative medicine and treatment of degenerative diseases. The uses of adult stem

cells, hES cells and iPS cells in regenerative medicine have the potential to extend for the treatment of many other health issues that so far lack effective treatments.

Adult Stem Cells and Human Embryonic Stem Cells

Stem cell biology marks its beginnings since the early 1900s when the term stem cell was first introduced by the Russian histologist Alexander Maximov. Today, stem cell biology is one of the most exciting areas of research and it continues to gain interest as application of this technology towards regenerative medicine and personalized therapeutic treatments continue to provide hope for treatment of degenerative diseases, tissue regeneration and creation of replacement organs for deficient hosts. Naturally occurring stem cells are divided in two categories: **adult stem cells** and **embryonic stem cells**.

Adult stem cells

Adult stem cells reside in the adult tissue, are multipotent and have the ability to differentiate into cell types of the tissue lineage where they are derived from. Work in bone marrow transplantation research was the foundation for the discovery of adult stem cells. Evidence from a 1945 case, studying several patients exposed to life-threatening doses of radiation showed that certain cells in the human body that had the potential to give rise to blood cells. Continuing investigative work in the bone marrow transplantation field resulted in the discovery of bone marrow stem cells (Becker et al., 1963). This was an important milestone that set the stage for the development of the stem cell biology field that we know and apply today (Figure 1.2). The study from Becker et al. was the first to confirm the presence of a certain population of cells in the bone marrow that demonstrated multipotency and could colonize the spleen of highly irradiated mice (Becker et al., 1963). This was the first finding confirming the existence of stem cells.

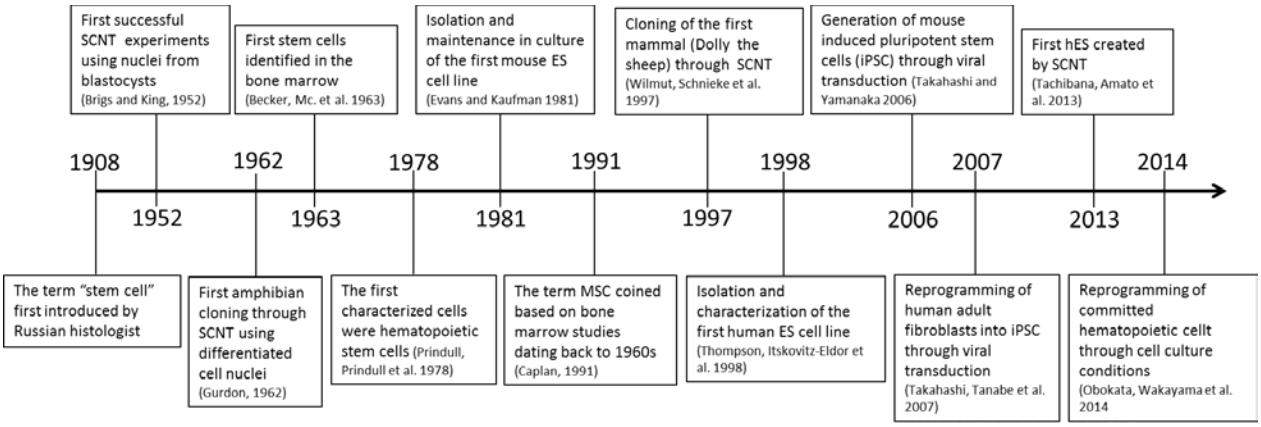

Figure 1.2 | **Abbreviated chronology of stem cell discoveries**

Similar to the study done by Becker et al. in 1963, Prindull et al. determined that cells isolated from umbilical cord blood had the potential to give rise to myelotic colonies in viscous suspension cultures (Prindull et al., 1978). Today, many types of adult stem cells have been identified; **mesenchymal stem cells** and **hematopoietic stem cells** isolated from the bone marrow (Abramson et al., 1977; Berenson et al., 1988; Minguell et al., 2001), multipotent hematopoietic stem cells isolated from **peripheral blood** (Takaue et al., 1989), multipotent adult stem cells isolated from the **dermis of the skin** (Toma et al., 2001; Toma et al., 2005), and multipotent **adipose stem cells** (Gimble and Guilak 2003; Zuk et al., 2002). In most cases adult stem cells are described as not able to cross developmental lineages, however several studies have shown that some adult stem cells like **neural stem cells** from the brain (Clarke et al., 2000) and bone marrow stem cells (Krause et al., 2001) can differentiate into cell types from different germ layers.

Embryonic stem cells

Embryonic stem cells are pluripotent cells that have unlimited ability for self-renewal and the potential to develop onto three germ layers formed during embryogenesis: endoderm, mesoderm, and ectoderm (Figure 1.3). The successful isolation and maintenance of the first embryonic stem (ES) cell lines was achieved from the inner cell masses of mouse blastocysts in the early 1980s (Evans and

Kaufman 1981; Martin 1981). Mouse embryonic stem cells provided the benchmark and established the generic requirements for isolation and maintenance of ES cells. A cell must have the following characteristics in order to be considered a true pluripotent stem cell; It can give rise to any cell type in the body; It is derived from a pluripotent cell population; it is stably diploid and karyotypically normal *in vitro*; It can be propagated indefinitely maintaining pluripotency; it can differentiate into cell types of all three embryonic germ layers, *in vitro* as well as into teratomas after grafting. It is also important for a stem cell line to be derived from a single cloned cell. This last criterion ensures that the stem cell is truly pluripotent, is responsible for the variety of differentiated cells derived and eliminates the possibility that the variety of differentiated cells derived comes from several multipotential cells present in culture. In 1998, Thompson was the first one to derive, maintain and fully characterize human embryonic stem cells in culture (Thomson et al., 1998). This important achievement opened the doors to the exploration of ideas of using human embryonic pluripotent cells for personalized cell based therapies, to treat diseases that come from the destruction of specialized cells that cannot be regenerated *in vivo*, that so far had presented a challenge in the medical field.

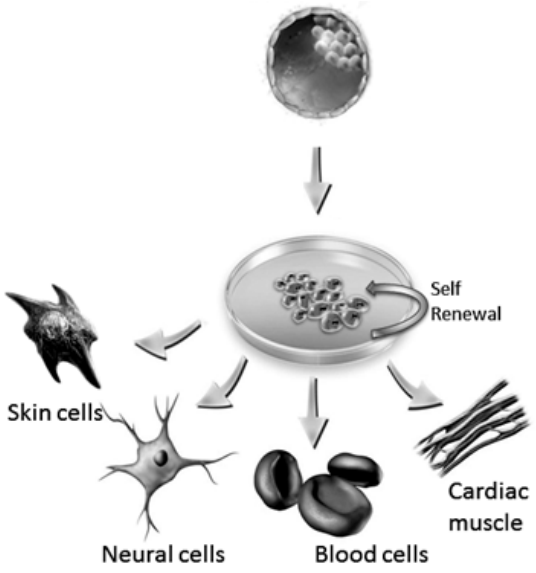

Figure 1.3 | **Embryonic stem cells derived from the inner cell mass of a blastocyst**
a. Embryonic stem cells are cells isolated from the inner cell mass of the blastocyst of the early embryo and have the ability to differentiate in three germ layers; the endoderm, mesoderm and ectoderm.
(Modified from http://www.stemcellresearchfoundation.org/WhatsNew/Pluripotent.htm)

Strategies for Reprogramming Differentiated Cells

A terminally differentiated cell was long thought to be in a stable state by permanently inactivating genes that it no longer needs thus losing the ability to alter its pattern of gene expression. More recent work has shown that the phenotypic state of the cell is controlled by dynamic mechanisms that can be altered, resulting in changes in gene expression patterns, a process referred to as epigenetic reprogramming. To date several approaches have been described that allow for epigenetic reprogramming and reversion of a differentiated cell phenotype to an embryonic state such as nuclear transfer, cell fusion, stem cell-factor transduction, and modification of culture conditions (Figure 1.4).

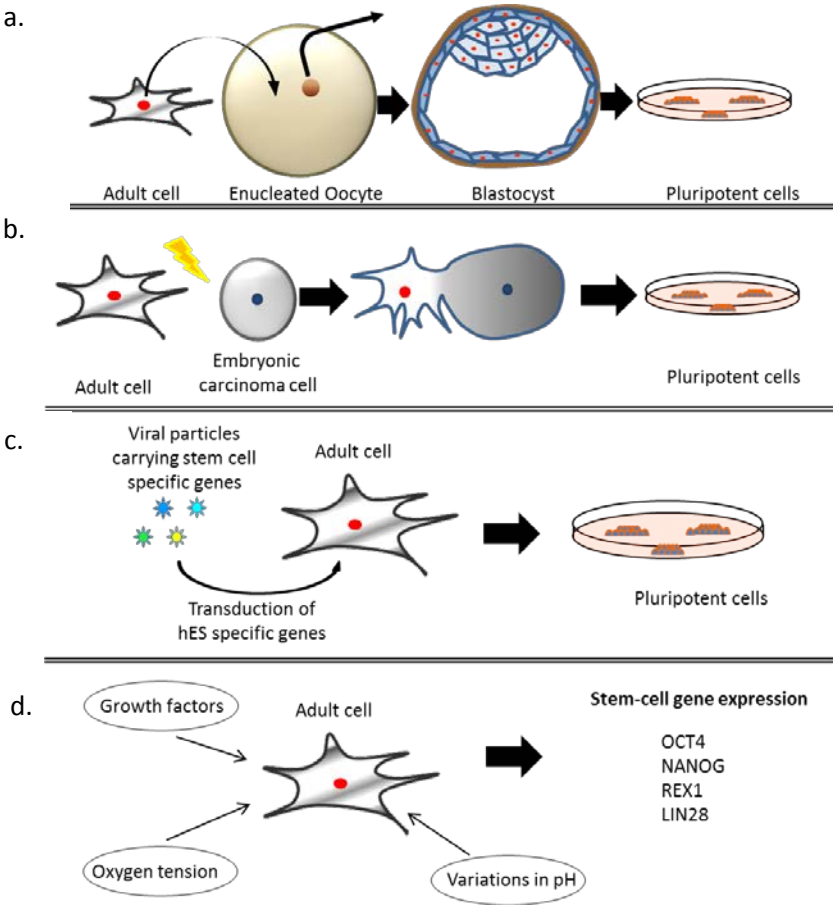

Figure 1.4 | **Strategies for reprogramming**
a. Nuclear transfer. The nucleus of a somatic cell can be transplanted into an enucleated oocyte and the somatic cell nucleus is reprogrammed so that the cells derived from it are pluripotent.
b. Cell fusion. Fusion of two distinct cell types to form a single entity. The resultant fused cells can be heterokaryons or hybrids. If the fused cells proliferate, they will become hybrids, and on division, the nuclei fuse to become 4*n* or greater. If the cells are derived from the same species, their karyotype will remain euploid however, if they are from different species, they will be aneuploid, as chromosomes will be lost and rearranged.
c. Stem-cell factor transduction. Induced pluripotent stem (iPS) cells can be generated through forced expression of stem cell specific genes in differentiated cells. iPS cells have similar properties to ES cells.
d. Culture condition modification. Expression of stem cell specific proteins can be induced in adult somatic cells through addition of growth factors and modification of oxygen tension. Reprogramming of mouse adult cells in iPS cells can be achieved through modification of pH conditions prior to culturing cells under stem-cell growth conditions.

Somatic cell nuclear transfer

Nuclear reprogramming work targeting reproductive cloning can be traced back to the mid-1900s, and the first successful nuclear transfer experiments showing nuclear reprogramming were published over 50 years ago by Briggs and King (Briggs and King 1952). They transferred nuclei from early blastocysts into enucleated oocytes which resulted in the birth of live *Rana pipiens* clones (Briggs and King 1952). Although their experiments resulted in swimming tadpoles, the investigators had difficulties reproducing their findings with nuclei from differentiated cells. This was the first report that paved the way to show that nuclei can change their gene expression profile when introduced in a new environment. These findings were later refined by John Gurdon. Gurdon used nuclei from *Xenopus* intestinal epithelial cells, which are cells in the final stage of differentiation of the epiderm in *Xenopus,* and was able to obtain completely normal male and female *Xenopus* clones (Gurdon 1962a; Gurdon 1962b). Gurdon's research was of critical importance because it was the first report to show cloning of a normal adult amphibian organism and that nuclei from a fully differentiated cell can be reprogrammed to change their gene expression profile. Gurdon's work set the stage for the ground breaking therapeutic cloning work that followed in the later years.

Oocytes and eggs have long been recognized as suitable environments for studying fundamental questions in cell biology, such as regulation of the cell cycle, DNA replication, chromatin remodeling, and transcription. While both developmental stages, immature oocytes (arrested at prophase of the first meiosis MI) and mature eggs (arrested at metaphase of the second meiosis MII) may be used as recipient cytoplasm, it is important to note that oocytes are actively engaged in transcription, while eggs are transcriptionally inactive and mainly engage in DNA replication after activation. It has been shown that nuclear components that are released from oocyte nuclei during maturation from MI to MII are required for successful reprogramming of somatic cell nuclei after nuclear transplantation, as removal of oocyte nuclei also removes nuclear components required for reprogramming (Miyamoto et al., 2009). In

1997, the first report was published showing cloning of the first mammal. Dolly the sheep was cloned through the fusion of a mammary epithelial cell with an enucleated oocyte (Wilmut et al., 1997). Several cloning strategies have demonstrated that nuclei from terminally differentiated cells, when introduced into mature eggs can be successfully reprogrammed, form embryos, and after transplantation into recipient animals develop to term (Egli et al., 2007; Wilmut et al., 1997).

Cell fusion

Cell fusion is the process of combining one mononuclear cell with another. This can be achieved through the application of an electrical field, use of chemicals such as polyethylene glycol, or viral based cell fusion through use of sendai virus. Developmental plasticity of somatic cell nuclei can be at least partially re-activated when nuclei are exposed to factors present in the cytoplasm of pluripotent cell types, such as embryonic stem cells (ES) and embryonic carcinoma cells (EC). For example, fusion of somatic cells with ES or EC cells leads to X chromosome reactivation within the heterokaryons (Tada et al., 2001), changes in gene expression profile (Cowan et al., 2005; Pereira et al., 2008; Zhou and Melton 2008), and acquisition of stem cell properties, including contribution to all germ layers in teratomas and in aggregation chimeras (Cowan et al., 2005; Pells et al., 2002; Tada et al., 1997; Tada et al., 2001; Terada et al., 2002; Ying et al., 2002).

Reprogramming through permeabilization

Treatment of reversibly permeabilized somatic cells with extracts of undifferentiated pluripotent ES or EC cells induces the expression of genes associated with pluripotency such as *Oct*4 and *Nanog*, causes down-regulation of somatic cell-specific genes such as *lamin A,* and enhances their *in vitro* differentiation capacity (Taranger et al., 2005). These modifications are observed at least temporarily after the resealing of permeabilized cells incubated in cell extracts (Collas and Taranger 2006). *Xenopus* oocyte extracts have been used extensively for their ability to affect chromatin structure (Dimitrov and Wolffe 1996), cell cycle and DNA replication (Lu et al., 1999) and gene expression in cultured

mammalian cells (Gurdon and Byrne 2003; Miyamoto et al., 2007). The cytoplasm of mature *Xenopus* oocytes alters chromatin structure through a series of DNA and DNA-binding protein changes that lead to expression of early embryonic developmental genes (Byrne et al., 2003; Freberg et al., 2007; Kimura et al., 2004; Taranger et al., 2005). Specifically, treatment of reversibly permeabilized somatic cells with complete *Xenopus* egg cytoplasmic extracts induces the expression of OCT4 (Byrne et al., 2003), NANOG (Koziol et al., 2007) and SOX2 (Miyamoto et al., 2007). While these very significant changes take place, there are no reports of attempts to culture and maintain the extract-reprogrammed cells for longer periods of time in culture. The *Xenopus* egg extract system offers an opportunity to identify cytoplasmic factors that have the ability to reprogram somatic cell nuclear memory. This thesis work investigates the composition and the ability of different extract fractions to reprogram nuclei of human dermal fibroblasts. We monitor the removal of somatic histone H1, reactivation of stem cell transcription factors, and their expression and localization to cell nuclei (Kole et al., 2014). We investigate the role of FGF2 not only in the induction of these changes, but its role in their maintenance over prolonged periods of time *in vitro*. Finally, we examine cells' potency by the ability to cross lineage differentiation boundaries upon select differentiation conditions and the ability to form teratomas in SCID mice.

Reprogramming with stem cell specific factors

Despite the potential that embryonic stem cells hold, they are met with mixed enthusiasm. The use of hES for therapies faces significant challenges related to their origin and derivation, as well as technical difficulties with growing ES cells in large enough numbers that they can be used for cell therapies. Work to overcome these challenges resulted in generation of iPS cells through over-expression of a defined set of factors using viral vectors (Takahashi and Yamanaka 2006), first from mouse fibroblasts in 2006, then a year later reprogramming of adult human fibroblasts into iPS (Takahashi et al., 2007) (Figure 1.4 c). Direct reprogramming of a differentiated cell into a developmentally more plastic cell offers an alternative to applications that cannot be addressed using

hES cells or adult stem cells. While the breakthrough generation of iPS through reprogramming with viral vectors provided new opportunities for personalized cell therapy, the field faced new challenges associated with the efficiency and method of reprogramming. Using viral vectors to reprogram somatic cells into an undifferentiated state is not suitable for therapeutic applications. Insertional mutagenesis from viral transduction can result in reactivation of the inserted viral genes later in development. Following the 2006 publication by Takahashi and Yamanaka, several studies were published addressing the problem of viral particle use for reprogramming. Thompson and colleagues reported the generation of iPS cells by transfection with non-integrating episomal vectors (Yu et al., 2009). The same year, a *piggyBac* transposon system (Kaji et al., 2009; Woltjen et al., 2009) and a Cre-recombinase excisable viruses system (Soldner et al., 2009) were reported. These systems allowed the transgenes to be excised using recombinant gene expression post reprogramming. These methods appeared to be more acceptable than viral reprogramming; however they can still introduce harmful alterations of the host cell as a result of residual sequences and chromosomal disruptions. A study published in 2009, showed that it is possible to reprogram differentiated human cells to an undifferentiated state using purified proteins fused to cell permeable peptides (Kim et al., 2009). This reprogramming method poses minimal clinical risk because it does not involve viral particles; however its reprogramming efficiency is much lower than the previously described reprogramming methods. The very low reprogramming efficiency introduces a challenge for generating pluripotent cells in large enough numbers that can be used for cell therapies in a timely fashion.

Reprogramming with defined culture conditions

In vitro culture conditions have been shown to play an important role in determining the fate of cultured cells (Gilbert et al., 2010; Kondo et al., 2000; Kubota et al., 2004). In 2009 we published work showing partial reprogramming of primary adult human fibroblasts through modification of culture conditions (Page et al., 2009). Primary skin fibroblasts cultured under low oxygen conditions (2-5% O_2)

and supplemented with exogenously added FGF2 activated translation of stem cell specific proteins OCT4, NANOG, SOX2 and REX1, and reached population doubling well above the Hayflick limit (Page et al., 2009). More recently, published work identified culture conditions that can fully reprogram committed hematopoietic cells to express stem cell marker OCT4 and acquire pluripotency (Obokata et al., 2014). Obokata et al. showed that low pH incubation of $CD45^+$ cells, followed by culturing under stem cell conditions can reprogram these cells to acquire pluripotency (Obokata et al., 2014). These findings are still being validated to determine whether they are reproducible and to determine the true potential of low pH incubation in reprogramming.

Stem Cell Culture

Over the past few decades, research in the embryonic stem cell field has shown that pluripotent stem cells have the ability to self-renew and differentiate into more specialized cells in the body and hold a lot of potential in regenerative medicine, treatment of degenerative diseases and drug discovery. A lot of research in the field is focused towards understanding the molecular mechanisms governing stem cell fate, but much of the effort has been focused on developing clinically compliant culture systems that allow for expansion and maintenance of pluripotent cells in an undifferentiated state. The first pluripotent stem cell culture systems included use of animal serum and mouse embryonic fibroblast feeder layers (Furue et al., 2008; Lu et al., 2006; Yao et al., 2006). These growth conditions were not suitable for clinical applications of pluripotent stem cells as they contained animal derived undefined products. One approach was to replace the mouse feeder layer with cells of human origin (Ellerstrom et al., 2006; Hovatta et al., 2003; Richards et al., 2002; Wang et al., 2005) or eliminate the feeder layer altogether and use surface coatings such as Matrigel™, that supported proliferation of hES cells in an undifferentiated state (Montes et al., 2009; Xu et al., 2001). Efforts to completely eliminate the need for animal derived products led to a publication that identified 30 factors secreted by the feeder layer that

supported growth of pluripotent stem cells (Chin et al., 2007). Later studies determined that several of the 30 identified secreted factors (FGF2, GABA, TGF-β, activin, Wnt1 and pipecolic acid) supported the proliferation of pluripotent stem cells in an undifferentiated state (Dvorak et al., 2005; Ludwig et al., 2006; Vallier et al., 2005). These studies determined that relatively high levels of FGF2 were required to support proliferation of pluripotent stem cells in an undifferentiated state (Chin et al., 2007; Ludwig et al., 2006; Olmer et al., 2010), and removal of FGF2 from the culture medium resulted in spontaneous differentiation of human pluripotent stem cell cultures (Eiselleova et al., 2009; Kjartansdottir et al., 2012). Addition of FGF2 in the culture medium has been shown to support single cell culturing and long term expansion of human pluripotent stem cells in an undifferentiated state (Valamehr et al., 2011). FGF2 is a required factor for expansion of human pluripotent stem cells in an undifferentiated state, and understanding the role and mechanism of action of FGF2 in supporting human pluripotent stem cell growth would allow for better understanding of how to control human pluripotent stem cell fate.

Fibroblast Growth Factor Family

Growth factors and the pathways they engage play an important role in controlling vital cellular programs in differentiated cells as well as pluripotent stem cells. Activation of mitogen activated protein kinase (MAPK) signaling through binding of FGF2 to FGF tyrosine kinase receptors, is directly involved in controlling the self-renewal and differentiation of human pluripotent stem cells (Dvorak et al., 2005; Li et al., 2007). It has been shown that FGF2 is a required factor regardless of the stem cell growth medium used; however, the molecular mechanisms by which FGF2 supports growth of human pluripotent stem cells in an undifferentiated state are not fully understood.

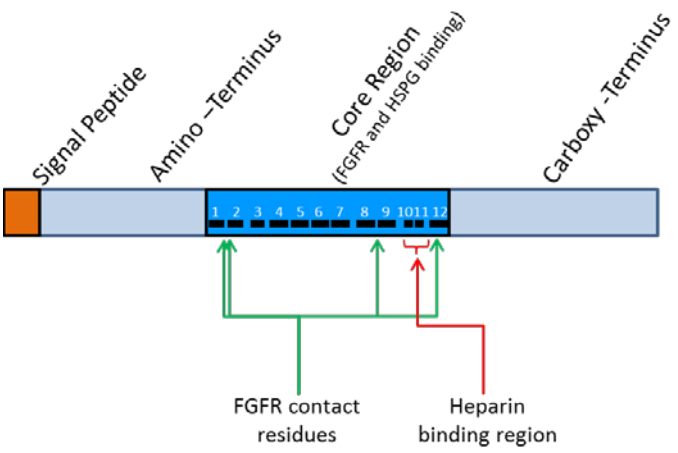

Figure 1.5 | **Generic structure of fibroblast growth factor family members**
Structure of generic FGF protein containing a signal sequence and the conserved core region which contains receptor and HSPG binding sites. The core region contains 12 β strands (black boxes) Green arrows indicate positions of FGFR contact. Red arrow indicates position of Heparin binding region. (modified from Ornitz and Itoh 2001)

FGF2 is a member of a large family of polypeptide growth factors found in many multicellular organisms. FGF2 is one of the first members of the FGF family discovered and was identified as a factor with the ability to stimulate proliferation of mouse 3T3 fibroblasts *in vitro* (Armelin 1973). FGF1 also acts as a mitogen for fibroblasts. FGF1 and FGF2 are considered to be the prototypic members of the FGF family. The human FGF family includes 22 members, FGF1-FGF23, all ranging between 150-300 amino acids and sharing a high degree of homology (Figure 1.5), with 30-60% identity in the conserved 120 amino acid core region (Itoh and Ornitz 2004; Ornitz and Itoh 2001). Phylogenetic analysis classifies FGF family members into seven subfamilies based on potential evolutionary relationship and gene location (Figure 1.6). FGF subfamily members are expressed in a tissue specific pattern and play different roles that are dependent on the stage of development where they are expressed.

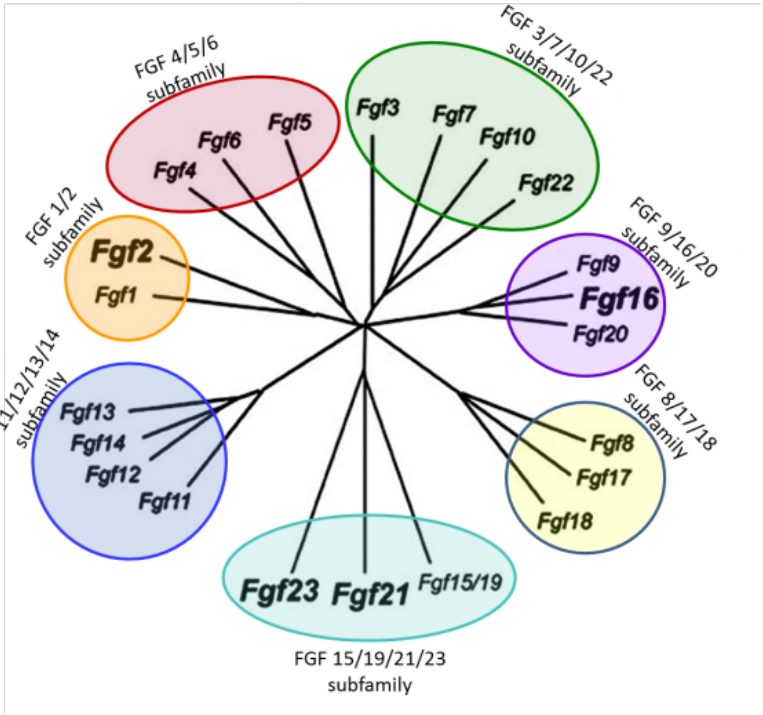

Figure 1.6 | **Phylogenetic classification of FGF family members**
This classification groups the members of FGF family in seven groups based on their gene location and potential evolutionary relationship (modified from Itoh and Ornitz 2011)

The human FGF family has the same number of members and their phylogenetic classification is the same as the mouse. Another classification scheme groups members of the FGF family by mechanism of action (Itoh and Ornitz 2011) into intracrine, paracrine, and endocrine function (Figure 1.7).

FGF family members play important roles during embryonic development as well as post-natally during nervous system development (Ford-Perriss et al., 2001), limb development (Li et al., 1996), injury response, wound repair (Itoh and Ornitz 2011) and angiogenesis (Lieu et al., 2011). Some FGF family members also contribute to disease development when missexpressed. Resulting phenotypes from disruption of *Fgf* genes by homologous recombination, in mice, range from embryonic lethality to changes in adult physiology (Table 1.1) (reviewed in (Itoh and Ornitz 2011)).

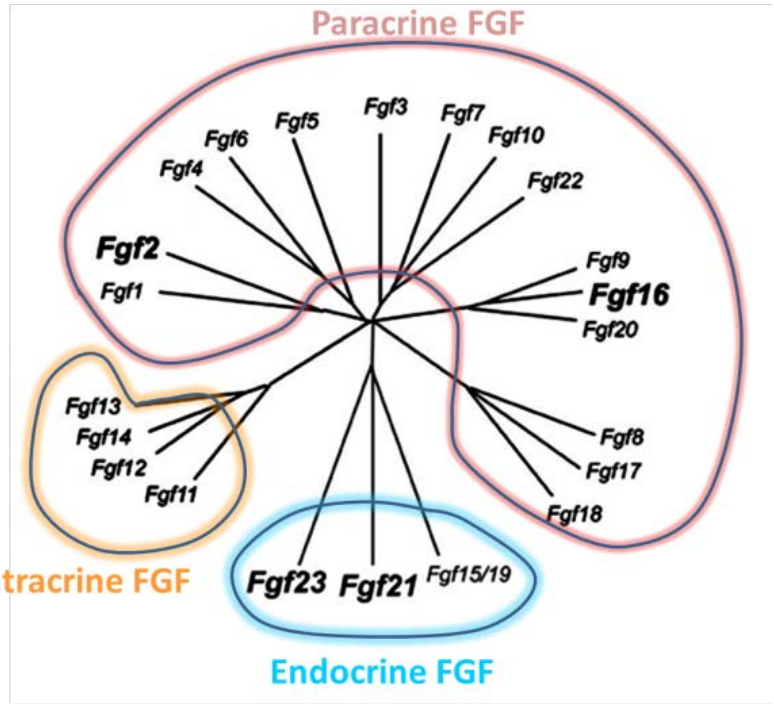

Figure 1.7 | **Functional classification of FGF family members** This classification groups the members of FGF family in three groups: paracrine, endocrine and intracrine, based on their mechanism of action (modified from Itoh and Ornitz 2011)

It is important to note that FGFs are able to control the expression of other genes in a dose-dependent manner (Green et al., 1992; Kengaku and Okamoto 1995). Eighteen of the 22 FGF family members act as ligands for high affinity FGF receptors which activate intracellular signaling pathways such as Ras-MAPK (Tsang and Dawid 2004), PI3-Kinase (Ong et al., 2001) and PLCY pathway (Klint et al., 1999). Four of the FGF family members (FGF11, FGF12, FGF13 and FGF14) do not bind and activate receptor tyrosine kinases and are thought of as FGF homologous factors (FHF) (Olsen et al., 2003). Their known role is regulation of the electrical excitability of neurons (Goldfarb et al., 2007; Xiao et al., 2007).

Table 1.1 | **Phenotypes related to targeted disruption of FGF genes**

Gene	Survival	Phenotype	Reference
FGF1	Viable	No obvious phenotype	(Miller et al., 2000)
FGF2	Viable	Abnormal neuronal, skeletal and skin phenotypes	(Dono et al., 1998)
FGF3	Viable	Abnormal Inner ear and tail outgrowth	(Mansour et al., 1993)
FGF4	Lethal E5.5	Inner cell mass proliferation	(Feldman et al., 1995)
FGF5	Viable	Long hair; "Angora"□ phenotype	(Hebert et al., 1994)
FGF6	Viable	Impaired muscle regeneration	(Floss et al., 1997)
FGF7	Viable	Hair follicle and kidney deficiency	(Guo et al., 1996)
FGF8	Lethal E8.5	Many phenotypes including gastrulation; brain; heart and craniofacial development	(Meyers et al., 1998)
FGF9	Lethal Po	Lung; XY sex reversal	(Colvin et al., 2001b) (Colvin et al., 2001a)
FGF10	Lethal Po	Many phenotypes including limbs; lungs; kidneys and others	(Min et al., 1998) (Sekine et al., 1999)
FGF14	Viable	Neurological phenotype-ataxia and a paroxysmal hyperkinetic movement disorder	(Wang et al., 2002)
FGF15	Viable	No defect in inner ear development; poor survival rate	(Wright et al., 2004)
FGF17	Viable	Midline cerebral development	(Xu et al., 2000)
FGF18	Lethal P1	Delayed ossification and increased chondrocyte proliferation; decreased alveolar spaces in the lung	(Liu et al., 2002) (Ohbayashi et al., 2002) (Usui et al., 2004)
FGF23	Viable	Hyperphosphatemia; hypoglycemia; reduced bone density and infertility	(Shimada et al., 2004)

Fibroblast Growth Factor 2 (FGF2)

FGF1 and FGF2 were the first FGF family members identified as mitogenic factors that supported the growth of NIH3T3 cells (Gospodarowicz 1974; Gospodarowicz 1975). Studies following Gospodarowicz's initial publications have so far identified 22 members of the FGF family, 18 of which part of a group of secreted polypeptides that use high-affinity membrane-spanning tyrosine kinase receptors as well as low affinity cell surface co-receptors. FGF2 is part of the secreted FGF family group that binds high affinity tyrosine kinase receptors. Five known isoforms of FGF2 are produced as a result of translation initiation at alternative start sites (Figure 1.8) ranging from 18kDa to 34kDa (Arnaud et al., 1999; Florkiewicz and Sommer 1989). The 18kDa secreted isoform is the low molecular weight (LMW) FGF2 while high molecular weight (HMW) FGF2 isoforms (22kDa, 22.5kDa 24kDa and 34kDa) are co-

linear extensions of the 18kDa isoform (Arnaud et al., 1999; Florkiewicz and Sommer 1989). The HMW isoforms are predominantly intracellular and can localize to cytoplasm as well as the nucleus, while the 18kDa isoform is mainly cytoplasmic and is secreted from cells through a process that is not well understood since it lacks a conventional secretion signal. Localization of FGF2 isoforms varies depending on the cellular condition and stimuli (Arese et al., 1999b) and HMW isoforms have been described in some occasions to be present in the extracellular space released from cells through a process known as vesicle shedding (Taverna et al., 2003).

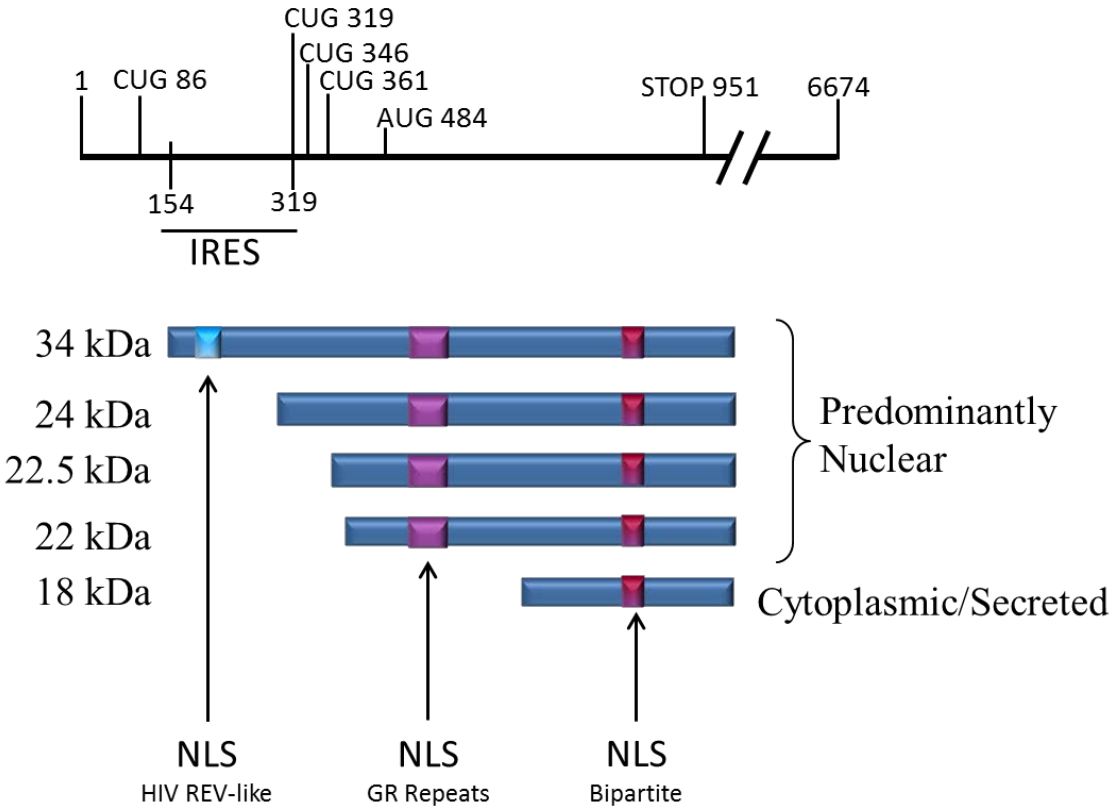

Figure 1.8 | **Human Fibroblast growth factor 2 (FGF2)**
Schematic representation of human FGF2 isoforms - One classical methionine initiation site (AUG) and four alternative leucine initiation codons (CUG). Five isoforms at the indicated molecular weights are produced as a result of translation initiation at alternative start sites. Four high molecular weight (HMW) isoforms are predominantly nuclear. The low molecular weight isoform is predominantly cytoplasmic/secreted. All FGF2 isoforms contain nuclear localization sequences (NLS) (modified from http://atlasgeneticsoncology.org/Genes/GC_FGF2.html)

Binding of FGF ligands to receptor tyrosine kinases results in the stimulation of various signal transduction cascades that have been implicated in multiple aspects of vertebrate and invertebrate embryonic development, tumor growth, angiogenesis, wound healing, and physiology (Ornitz and Itoh 2001; Ornitz and Marie 2002; Powers et al., 2000). Unregulated expression of FGFs has been shown to cause cancer as well as be involved in cancer progression (Dailey et al., 2005; Ezzat and Asa 2005; Grose and Dickson 2005). The low molecular weight (LMW) 18kDa isoform is the best studied, and is reported to have intracrine, autocrine as well as paracrine effects (Arese et al., 1999a; Strutz et al., 2000). Binding of the FGF2 ligand to the receptor triggers receptor dimerization, phosphorylation of its kinase domain, and activation of several intracellular kinase pathways such as Ras-MAPK, PLCϒ, PI3K (Figure 1.9).

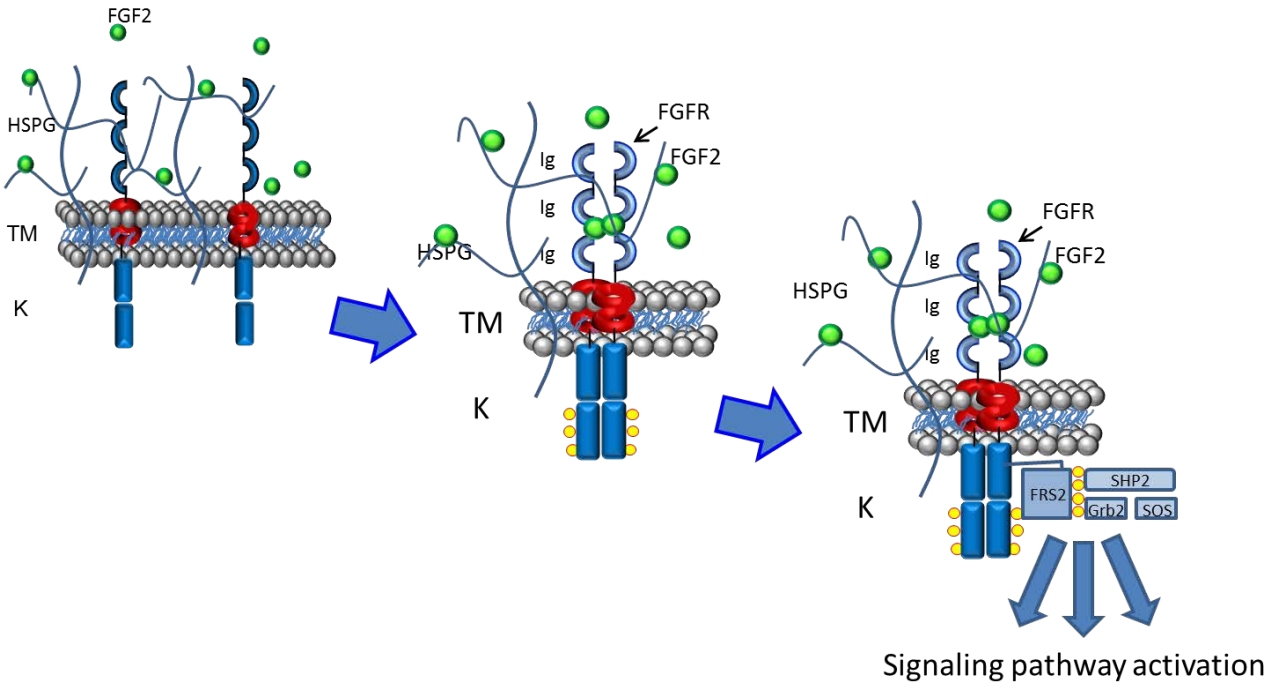

Figure 1.9 | **FGF2 binding to receptor tyrosine kinases**
Formation of the FGF ligand/HSPG/FGFR complex promotes receptor dimerization followed by receptor autophosphorylation at tyrosine residues and assembly of protein complexes that trigger activation of downstream signaling pathways

Previous research has demonstrated that HMW FGF2 isoforms can be transported to the extracellular environment (Santiago et al., 2011) and that the 24kDa FGF2 isoform when administered

exogenously to endothelial cells induces cell proliferation at a similar dose response to the 18kDa isoform (Taverna et al., 2003). Investigation of the ability and specificity of each isoform to bind various FGFRs and activate downstream kinase pathways is critical for understanding the biological mechanisms involved in normal vertebrate development and pathogenesis. Preceding studies have identified FGF2 as an important factor in the maintenance of pluripotency in human stem cells (Eiselleova et al., 2009; Zoumaro-Djayoon et al., 2011). FGF2 has been shown to promote self-renewal in hES cells by two potential mechanisms: by directly activating the MAPK pathway (Dvorak et al., 2005; Li et al., 2007) and by indirectly modulating TGFβ and Activin A signaling on the feeder layer, which supports hES cells self-renewal (Eiselleova et al., 2008; Greber et al., 2007).

In addition to its importance in supporting human pluripotent stem cell expansion in an undifferentiated state, FGF2 has also been involved in activation of stem cell pathways in already differentiated cells. Our lab has shown that the 18kDa FGF2 isoform, in combination with low oxygen conditions (2-5% oxygen), induces expression of stem cell specific genes and proteins in human dermal fibroblasts (Page et al., 2009). These findings are in conflict with reports which show that FGF2 stimulates the differentiation of mesoderm derived cardiovascular progenitors (Yang et al., 2008), trophoectoderm differentiation (Schenke-Layland et al., 2007), and differentiation of endoderm derived pancreatic cells (Funa et al., 2008). It has been suggested that one possible explanation to these very different outcomes could be that FGF2 has different effects on differentiating cells compared to pluripotent cells (Debiais et al., 1998). When FGF2 was supplemented to different cell types, it was shown that FGF2 signaling varied depending on the type of cell (Kinkl et al., 2001). The multifunctional nature of FGF2 with respect to dosage, cell type and developmental stage makes FGF2 an important target for study.

Fibroblast Growth Factor Receptors

High Affinity FGF Receptor Tyrosine Kinases

FGFs mediate their biological activity through the activation of surface FGF receptor (FGFR) tyrosine kinases (Ornitz et al., 1996; Zhang et al., 2006) and possess broad mitogenic and angiogenic activity. Interaction of extracellular ligands with receptor tyrosine kinases can direct stem cells toward self-renewal or differentiation into different lineages depending on the interaction specificity between the ligand and the receptor. Receptor tyrosine kinases are transmembrane receptors that translate extracellular signals into the activation of intracellular pathways and can be of high importance in deciding cell fate (Table 1.2).

Table 1.2 | Phenotype related to targeted disruption of FGFR genes

Gene	Survival	Phenotype	Reference
FGFR1	Lethal, E9.5–E12.5	Defective cell migration through primitive streak; posterior axis defect	(Deng et al., 1994; Yamaguchi et al., 1994)
FGFR1 IIIb	Viable	No obvious phenotype	(Partanen et al., 1998)
FGFR1 IIIc	Lethal, E9.5	Defective cell migration through primitive streak; posterior axis defect	(Partanen et al., 1998)
FGFR2	Lethal, E10.5	Defect in placenta and limb bud formation	(Xu et al., 1998)
FGFR2 IIIb	Lethal, P0	Agenesis of lungs, anterior pituitary, thyroid, teeth and limbs	(De Moerlooze et al., 2000)
FGFR2 IIIc	Viable	Delayed ossification, proportionate dwarfism, synostosis of skull base (chondrocranium)	(Eswarakumar et al., 2002)
FGFR3	Viable	Bone over growth; inner ear defect	(Colvin et al., 1996; Deng et al., 1996)
FGFR4	Viable	No obvious phenotype; growth retardation and lung defects in FGFR3 null background	(Weinstein et al., 1998)

The general FGF receptor structure includes a split tyrosine kinase (TK) domain responsible for catalytic activity, and the juxtamembrane domain responsible for binding of intracellular adapter proteins. A single transmembrane (TM) domain is present and is responsible for anchoring the receptor and maintaining conformation required for ligand dependent activation. Depending on the receptor

variant, two or three immunoglobulin (Ig) domains are responsible for ligand binding and ligand specificity. The acidic box and heparin binding domain are required for heparin binding and extracellular matrix (ECM) interactions (Figure 1.10). Interaction of different FGF ligands with receptor tyrosine kinases can stimulate very diverse biological responses in a wide variety of developmental stages, cell types and organisms (reviewed in (Bottcher and Niehrs 2005; Itoh and Ornitz 2004; Itoh and Ornitz 2011; Katoh and Nakagama 2013; Ornitz and Itoh 2001; Presta et al., 2005).

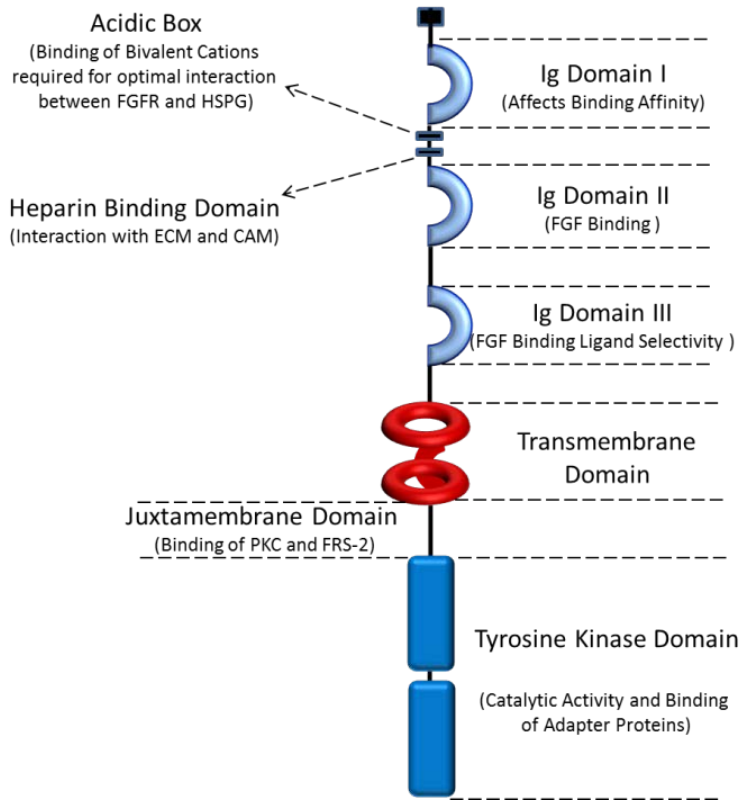

Figure 1.10 | **Generic structure of FGFR proteins**
Schematic representation of the main structural features of FGFR proteins: A split TK domain responsible for catalytic activity, Juxtamembrane domain responsible for binding of intracellular adapter proteins, TM domain responsible for anchoring of the receptor and maintaining conformation required for ligand dependent activation, Ig domains responsible for ligand binding and specificity, acidic box and heparin binding domain required for heparin binding and extracellular matrix (ECM) interactions

Four genes encoding FGF receptor tyrosine kinases have been identified which code for several FGF receptor variants. The first FGF receptor (FGFR1) was identified in chicken embryos as a 130kDa transmembrane protein with the ability to bind FGF2 (Lee et al., 1989). Subsequent studies described four unique human cDNA clones encoding multiple receptor forms that demonstrate identity in some regions but were highly diverse in select regions of the extracellular domain (Johnson et al., 1990;

Johnson et al., 1991). Based on the cDNA sequences, these studies classified FGF receptors in two classes: long and short. At the amino acid level, the long forms differ from each other in the number of Ig domains they contain, showing that the "long" receptors contain three Ig domains while the "short" receptors contain two Ig domains (Johnson et al., 1991). Regardless of the number of Ig domains, both types of receptors are capable of binding FGF1 and FGF2 to initiate a biological response.

A key feature of FGF receptor tyrosine kinases is that for FGFR 1-3, several receptor variants can be generated from the same gene by alternative RNA splicing (Givol and Yayon 1992) (Figure 1.11).

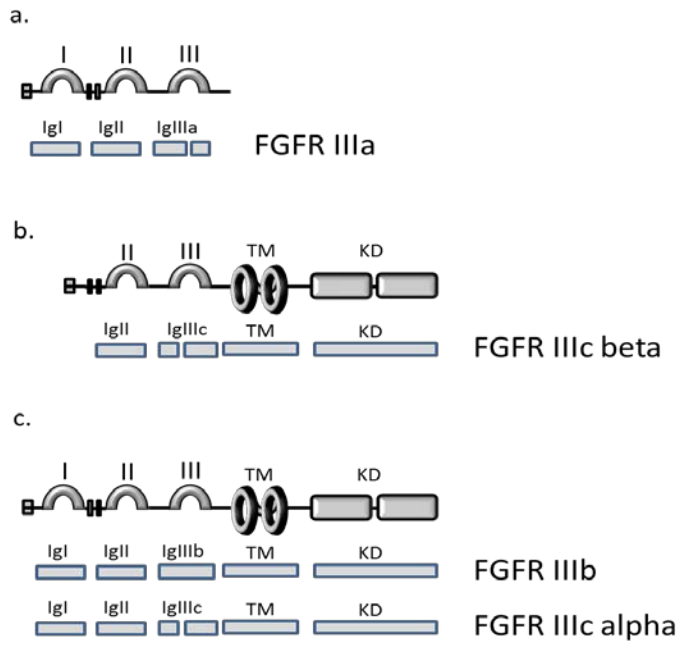

Figure 1.11 | **Schematic representation of soluble and membrane spanning FGF receptor variants**
a. FGF receptor IIIa lacking the TM domain and the kinase domain as a result of RNA splicing. This receptor variant translates exon IIIa in the third Ig domain.
b. FGF receptor IIIc beta lacking the first Ig domain. This receptor variant translates exon IIIc in the third Ig domain. Lack of the first Ig domain has not been reported to affect activity of this receptor variant.
c. FGF receptor IIIb and FGF receptor IIIc alpha. These receptor variants have three Ig domains and differ from each other in the third Ig domain. Receptor variant IIIb translates exon IIIb and receptor variant IIIc translates exon IIIc respectively. Differences in the third Ig domain have been reported to affect ligand binding specificity

Two transmembrane variants are produced for each of the FGFRs 1-3, coming from alternative exon usage in the third Ig domain. These variants, named IIIb and IIIc alter the ligand binding IgIII domain of the receptor (Zhang et al., 2006) and are most likely responsible for variations in FGFR ligand binding specificity. Another variant of the receptor, classified as FGFR IIIa, is produced as a result of alternative RNA splicing and is secreted from of the receptor. This secreted variant of FGFR lacks both the transmembrane as well as the intracellular kinase domain. This variant has the ability to bind FGF

ligands; however it demonstrates no known biological activity (Johnson et al., 1991). A theory for the existence of the secreted receptor forms is to regulate levels of FGF in the extracellular space. This mechanism has been described previously for a secreted form of the interleukin 4 (IL-4) receptor (Mosley et al., 1989).

Low Affinity FGF Receptors

Biochemical studies indicate that in addition to high affinity interactions with receptor tyrosine kinases, FGF ligands are also involved in important interactions with low affinity co-receptors which include heparin and heparan sulfate proteoglycans (HSPG) (Mason 1994; Ornitz et al., 1995; Ornitz et al., 1992; Schlessinger et al., 1995). The FGF ligands and FGF receptors have binding sites for HSPGs and it is believed that HSPGs facilitate the binding of FGF to the receptor, mediate receptor dimerization and also provide protein stability and protect FGF from degradation (DiGabriele et al., 1998; Ornitz et al., 1992; Saksela et al., 1988; Spivak-Kroizman et al., 1994; Venkataraman et al., 1996). HSPGs are also thought to behave as natural reservoirs that can store FGF and provide cells with a prolonged supply of FGF even in situations of very short ligand exposure (Flaumenhaft et al., 1989; Saksela et al., 1988; Saksela and Rifkin 1990). In addition to providing stability and prolonged supply of FGF, HSPGs have also been shown to be directly involved in biological activities involving FGF2 (Roghani and Moscatelli 1992; Rusnati et al., 1993). In a subsequent study, Quarto and Amalric showed that FGF2 internalization and nuclear translocation occurs through binding of FGF2 to HSPGs, (Quarto and Amalric 1994). Moreover, they showed that binding of FGF2 to HSPGs activates intracellular pathways even in the absence of high affinity FGF receptor tyrosine kinases (Quarto and Amalric 1994).

Fibroblast Growth Factor signaling

Fibroblast growth factor signaling is an intricate network that involves 22 FGF family members, four high affinity FGF receptor tyrosine kinases with multiple variants as well HSPG low affinity non-

tyrosine kinase receptors, such as heparin, capable of generating signaling independently or in concert with high affinity FGF receptors. FGF signaling through high affinity FGF tyrosine kinase receptors is also known as canonical FGF signaling, while signaling not involving receptor tyrosine kinases is identified as non-canonical signaling.

Canonical FGF2 Pathway

The canonical FGF signal transduction pathway is dependent on activation of high affinity tyrosine kinase receptors and starts with binding of the FGF ligand to the receptor, in the presence of HSPGs, which triggers receptor dimerization and subsequent phosphorylation of the kinase domain (Yayon et al., 1991). Phosphorylation of multiple tyrosine residues on the kinase domain of the receptor triggers recruitment of several adaptor and docking proteins which result in phosphorylation and activation of different signaling pathways (Figure 1.12) [reviewed in (Schlessinger 2000)]. The best studied FGF signaling pathways are the Ras-MAP kinase pathway, the PI3 kinase – AKT pathway and the PLCϒ pathway.

The mitogenic Ras-MAP kinase pathway is the most common pathway activated by FGF ligands. Upon receptor dimerization and phosphorylation of its kinase domain, fibroblast substrate 2-α (FRS2α) gets phosphorylated and acts as a docking site for SH2 domain-containing proteins Grb2 and Shp2 (Dhalluin et al., 2000; Ong et al., 2000). Binding of the guanine nucleotide exchange factor SOS to this complex activates Ras which results in activation of Erk1/2, p38 and JNK MAP kinase pathways (Figure 1.12).

The PI3 kinase – AKT pathway is also dependent on FRS2 phosphorylation, which results in the formation of another multi-protein complex FRS2-GRB2-GAB1. Formation of this multiprotein complex leads to activation of PI3 kinase (Lamothe et al., 2004) (Figure 1.12). The FGF2-induced PI3 kinase – AKT pathway in endothelial cells is one of the most important cell survival pathways (Partovian and Simons 2004).

Unlike the Ras-MAP kinase and PI3 Kinase pathways, activation of the PLCY pathway does not require FRS2 phosphorylation and recruitment of multi-protein complexes (Figure 1.12).

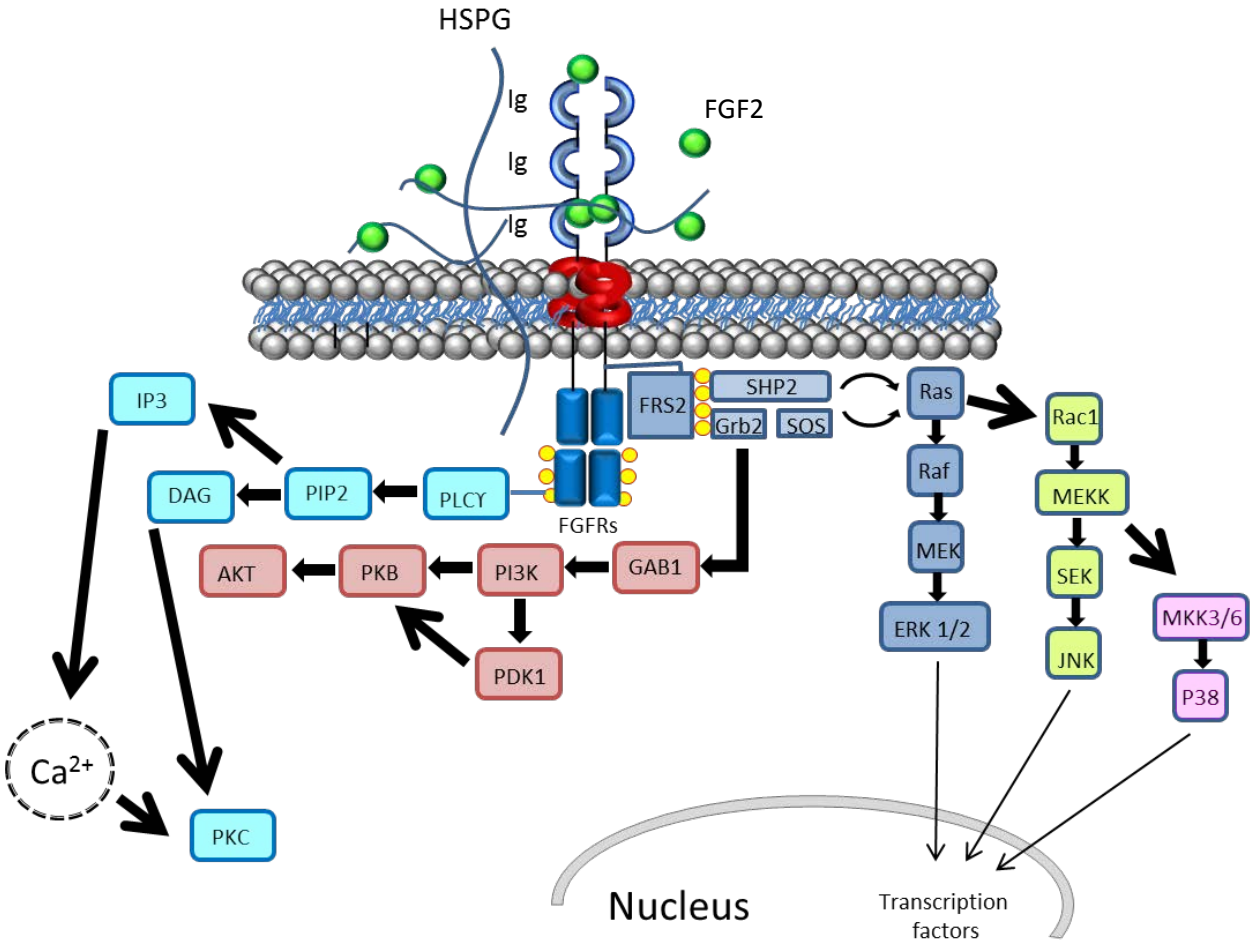

Figure 1.12 | **Schematic representation of FGF2 signaling pathways**
Binding of FGF ligands to high affinity receptor tyrosine kinases results in activation of several intracellular pathways with vital importance for cell survival, migration and proliferation. Shown above are the most well described intracellular pathways activated by FGF ligands.

Phosphorylation of the FGF receptor tyrosine kinase on Tyr766 provides a binding site for the SH2 domain of PLCY (Mohammadi et al., 1991). This results in production of diaglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), which releases intracellular calcium and results in activation of calcium dependent protein kinase C (PKC) family members.

Non-Canonical FGF2 pathway

The non-canonical FGF2 pathway functions independent of activation of receptor tyrosine kinases. One of the non-canonical FGF signaling pathways involves syndecan, a member of the HSPG family which demonstrates low affinity binding for FGF through their extracellular heparin sulfate chains. Syndecans can directly bind FGF ligands but have been originally classified as co-receptors whose FGF binding is a pre-requisite for FGF binding to receptor tyrosine kinases (Ornitz et al., 1995; Ornitz et al., 1992). HSPGs demonstrate FGF signaling ability beyond just acting as co-factors for receptor tyrosine kinase activation. Introduction of a mutation in syndecan-4 cytoplasmic domain, which disrupts PIP_2 binding, results in endothelial cells that are unresponsive to FGF2 (Horowitz et al., 2002) and lack of syndecan-4 cytoplasmic tail disrupts FGF signaling (Volk et al., 1999). Other studies have implicated syndecan-4 in FGF2 internalization in endothelial cells (Tkachenko et al., 2004). These findings are relatively new and more work is ongoing to determine the involvement of HSPGs in the transport of FGFs inside the cell as well as their nuclear translocation.

Objectives and Organization of Thesis

The overall goal of this project was to identify reprogramming factors in the cytoplasmic fraction of *Xenopus laevis* egg extract that would allow for the generation of safe, clinically relevant, autologous, multipotent cells. We also aimed to understand the role of exogenously added FGF2 in the reprogramming of human somatic cells by *Xenopus laevis* egg extract, how pathways involving FGF2 control the maintenance of stem cells in an undifferentiated state and how they affect differentiation of pluripotent/multipotent cells *in vitro*.

We hypothesized that *Xenopus laevis* cytoplasmic egg extract contains select fractions with reprogramming potential that in concert with exogenously supplemented fibroblast growth factor 2 can reprogram somatic cells into a multipotent state.

In this study, multipotency was defined as the ability of a cell to activate and maintain gene expression patterns similar to the ones observed in embryonic stem cells as well as gain the ability to differentiate and give rise to other cell types. The expected outcome of this project was the identification of select fractions of *Xenopus* cytoplasmic egg extract that, in the presence of exogenous FGF2, have the ability to reprogram differentiated cells into a multipotent state allowing generation of a transgene-free reprogramming method.

In order to systematically test this hypothesis, this thesis was organized in four objectives.

Objective I. Identification of *Xenopus laevis* egg extract components with potential activity to reprogram differentiated human dermal fibroblasts

(Kole, Ambady et al, 2014 Cell Reprogram 16(1): 18-28)

The development of induced pluripotent stem cells was a major step for the development of autologous stem cell therapies, but the clinical application of these cell therapies faces difficulties associated with quality and clinical compliance. To explore new methods for the generation of clinically compliant, autologous multipotent cells, chapter two of this thesis describes our efforts in identifying select *Xenopus laevis* cytoplasmic egg extract fractions with the potential to reprogram human somatic cells into an undifferentiated state. A transgenic cell line expressing Histone H1.2 fused to green fluorescent protein (GFP) was established to monitor nuclear reprogramming events in real time. To identify select fractions with reprogramming activity, size exclusion chromatography was used to fractionate total cytoplasmic extract, and various combinations of fractions were tested for reprogramming activity. The reprogramming potential of select fractions was monitored by observation

of nuclear reprogramming events in real time, assaying the expression of stem cell specific genes and proteins, and evaluating potential of reprogrammed cells in differentiation assays.

Objective II. Role of FGF2 isoforms in nuclear reprogramming, induction of stem cell specific genes and proteins in terminally differentiated cells, and the maintenance of human embryonic stem cells in an undifferentiated state.

(Kole et al. in preparation)

FGF2 has been described as a factor of vital importance in the maintenance of pluripotency in human ES cells. Five isoforms of FGF2 protein exist ranging from 18kDa to 34kDa, and while the 18kDa isoform is the most studied, it is also the most common isoform present in the extracellular space to signal cells. In chapter three of this thesis we describe our efforts in studying the role of high molecular weight FGF2 isoforms on the maintenance of pluripotency in human ES cells and their ability to induce the expression of stem cell specific genes in terminally-differentiated cells. By individually overexpressing and purifying each isoform we were able to test their activity and semi-quantitatively compare activity levels between all isoforms. Biological activity testing of each isoform was performed to show that HMW FGF2 isoforms can bind high affinity receptor tyrosine kinases, activate intracellular downstream pathways, maintain hES in an undifferentiated state and can induce expression of stem cell specific proteins in terminally differentiated cells with similar activity as the 18kDa FGF2. The findings that HMW FGF2 isoforms behave in a similar manner and activate the same pathways as the 18kDa isoform when found in the extracellular space support the hypothesis that HMW FGF2 isoforms are secreted in the extracellular space to potentiate the 18kDa FGF2 effect under certain conditions.

Objective III. Investigation of the interaction of FGF2 isoforms with FGF receptor tyrosine kinase variants

(Kole et al. in preparation)

There are 22 members in the FGF family, 18 of which act through 4 high affinity receptor tyrosine kinases. This organization allows for a high degree of ligand-receptor promiscuity, but at the same time offers a high degree of signaling diversity as a result of specific ligand-receptor interactions. Research has shown that interaction of the same ligand with different receptors or different ligands with the same receptor can have very different results on cell fate. In chapter 4 of this thesis we quantitatively investigated the binding of each FGF2 isoform to specific FGF receptor tyrosine kinases and their ability to activate the mitogenic pathway. FGF receptor variants were individually overexpressed in BaF3 cells (an IL-3 dependent murine pro-B cell line) and FGF2 isoforms were tested for their ability to support the growth of these cells in the absence of IL-3. FACS analysis following BrdU incorporation assays demonstrated that HMW FGF2 isoforms can all bind select FGF receptor tyrosine kinases and support the growth of BaF3 cells in the absence of IL-3. Chapter 4 also statistically analyzes these results to determine if there is a statistically significant difference in the way different FGF2 isoforms support growth of these cells. The results from this work, once again support the hypothesis that HMW FGF2 isoforms behave similarly to 18kDa isoform when found in the extracellular space.

Objective IV. Identification of nucleic acid binding targets for specific FGF2 isoforms

FGF2 is detected in the nucleus, cytoplasm as well as the extracellular space of expressing cells. HMW FGF2 isoforms have nuclear localization sequences that allow their accumulation in the nucleus. 18kDa FGF2 is mostly found in the extracellular space and cytoplasm of producing cells but has also been described to translocate into the nucleus. Chapter 5 of this thesis describes our work to identify nucleic acid binding targets for nuclear localized FGF2 isoforms. After over-expression of His-tagged

FGF2 isoforms, chromatin immunoprecipitation (ChIP) assays were performed with Ni-NTA resin, followed by Topo® cloning and sequencing of isolated DNA fragments. Targets identified by sequencing were then tested using electro-mobility shift assay (EMSA) to confirm the validity of the ChIP-Seq results. In chapter 5 of this thesis we report on a newly identified target for the 22.5kDa FGF2 isoform. Identification of more direct binding targets for FGF2 isoforms will allow for better understanding of their intracellular activity, and can be used to better control growth conditions and cell fate.

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Abstract

Direct reprogramming of a differentiated somatic cell into a developmentally more plastic cell type would offer an alternative to applications in regenerative medicine therapies that currently depend on ES cells, adult stem cells or iPS cells. Here we report the potential of specific *Xenopus laevis* egg extract fractions, in combination with exogenous FGF2, to affect the life span, morphology, gene expression, protein translation and cellular localization of OCT4 and NANOG transcription factors, and the developmental potential of human dermal fibroblasts *in vitro*. A gradual change in morphology is accompanied by the translation of embryonic transcription factors and their nuclear localization and a life span exceeding 60 population doublings. Cells acquire the ability to follow adipogenic, neuronal and osteogenic differentiation under appropriate induction conditions *in vitro*. Analysis of active extract fractions reveals that, *Xenopus* egg protein and RNAs as well as exogenously supplemented FGF2 are required and sufficient for induction of and maintenance of this phenotypic change. Factors so far identified in the active fractions include FGF2 itself, TGFβ-1, maskin, and nucleoplasmin. The identification of critical factors needed for reprogramming may allow for the non-viral, chemically defined derivation of human induced multipotent cells that can be maintained by exogenous FGF2.

Introduction

Mammalian oocytes and eggs have long been recognized as suitable environments for studying fundamental questions in cell biology, such as the regulation of the cell cycle, DNA replication, chromatin remodeling, and transcription. While both developmental stages, immature oocyte (arrested at prophase of the first meiosis MI) and mature egg (arrested at metaphase of the second meiosis MII) may be used as recipient cytoplasm, it is important to remember that oocytes are actively engaged in transcription (Gall and Callan 1962; Scheer et al., 1976), while eggs are transcriptionally inactive but able to support DNA replication after activation (Blow and Laskey 1986). It has been shown that nuclear

components that are released from oocyte nuclei during maturation from MI to MII are required for the successful reprogramming of somatic cell nuclei after nuclear transplantation, as removal of oocyte nuclei also removes nuclear components required for reprogramming (Hansis et al., 2004; Kikyo et al., 2000). Several cloning strategies have demonstrated that nuclei from terminally differentiated cells, when introduced into mature eggs can be successfully reprogrammed, form embryos, and after transplantation into recipient animals develop to term (Wilmut et al., 1997).

Developmental plasticity of somatic cell nuclei can also be at least partially re-activated when nuclei are exposed to factors present in the cytoplasm of pluripotent cell types, such as ES cells and EC cells. For example, the fusion of somatic cells with ES or EC cells leads to X chromosome reactivation within the hybrids (Tada et al., 2001), changes in gene expression profile (Pereira et al., 2008) (Cowan et al., 2005; Zhou and Melton 2008), and acquisition of stem cell properties, including contribution to all germ layers in teratomas and in aggregation chimeras (Cowan et al., 2005; Pells et al., 2002; Tada et al., 1997; Tada et al., 2001; Terada et al., 2002; Ying et al., 2002).

The treatment of reversibly permeabilized somatic cells with extracts of ES or EC cells induces the expression of genes associated with pluripotency, such as *OCT4*, *NANOG* and *SOX2*, causes down-regulation of somatic cell-specific genes, such as *lamin A*, and enhances their *in vitro* differentiation capacity (Taranger et al., 2005). These modifications are observed at least temporarily after the resealing of permeabilized cells incubated in cell extracts (Collas and Taranger 2006). In addition to mammalian systems, *Xenopus* oocyte extracts have been used extensively for their ability to affect chromatin structure (Dimitrov and Wolffe 1996), cell cycle and DNA replication (Lu et al., 1999) and gene expression in cultured mammalian cells (Gurdon and Byrne 2003; Miyamoto et al., 2007), demonstrating conservation of molecular regulatory mechanisms across species. The cytoplasm of mature *Xenopus* oocytes alters chromatin structure through a series of DNA and DNA-binding protein changes that lead to expression of early embryonic and developmental genes (Byrne et al., 2003; Freberg et al., 2007;

Kimura et al., 2004; Taranger et al., 2005). Specifically, the treatment of mammalian somatic cells with complete *Xenopus* egg cytoplasmic extracts induces the expression of *OCT4* (Byrne et al., 2003), *NANOG* (Koziol et al., 2007), and *SOX2* (Miyamoto et al., 2007). While these very significant changes take place, there are no reports of attempts to maintain the extract-reprogrammed cells for longer periods of time in culture (over 20 days).

The *Xenopus laevis* egg extract system offers an opportunity to identify cytoplasmic factors that have the ability to reprogram somatic cell nuclear memory. Here we investigate the composition and the ability of different extract fractions to reprogram nuclei of adult human dermal fibroblasts. We monitor the effects various fractions of extract on the removal of somatic histone H1, reactivation of stem cell transcription factors, and their expression and localization to cell nuclei. We investigate the role of FGF2 in not only induction of these changes, but its role in their maintenance over prolonged periods of time *in vitro*. Finally, we examine cells' potency by the ability to cross lineage differentiation boundaries upon select differentiation conditions and the ability to form teratomas in SCID mice.

Materials and Methods

Cell Culture

Primary adult human dermal fibroblasts (CRL #2352, American Type Culture Collection) were obtained at passage p3. Cells were expanded in DMEM/F12, supplemented with 10% fetal calf serum (FCIII, Hyclone), and 4 mM L-glutamine (MediaTech). Cultures were passaged at 80% confluence using standard procedures. Ten days prior to electroporation, serum concentration in culture medium was gradually decreased to 0.5% in order to induce accumulation of cells in G_1/G_0. After electroporation, cells were seeded into 24-well plates at 2,000 cells per well and grown in DMEM/F12, supplemented with 10% FC III, 2 mM L-glutamine, with or without 4 ng/ml FGF2 (PeproTech). The number of population doublings was calculated as $\log_2$ (#final/#initial). All cultures were incubated at 37°C, 5% O_2,

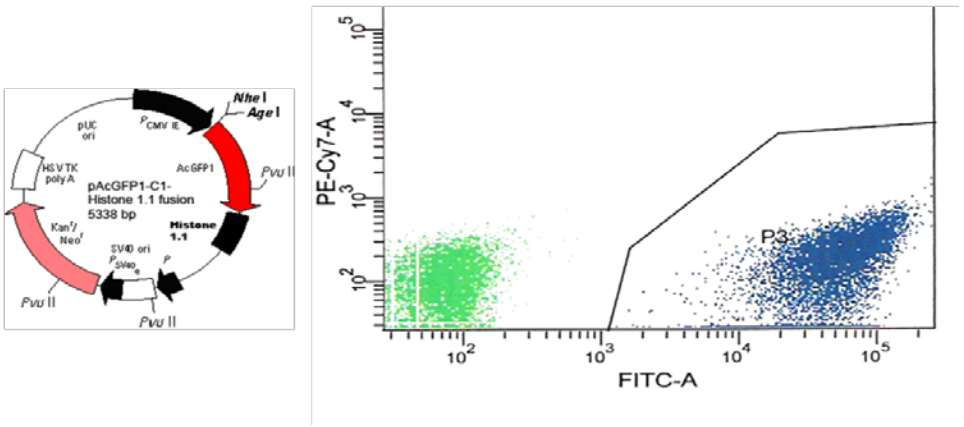
5% CO_2 and high humidity. Teratocarcinoma cells (NCCIT, American Type Culture Collection) were grown as recommended by the supplier. A sample of control and extract treated (electroporated) cells was incubated with 10 µM BrdU (Invitrogen) for 24 hours, washed and fixed in ice-cold methanol. DNA was denatured with 1N HCl and cells incubated with mouse monoclonal anti-BrdU-AlexaFluor 488-conjugated antibody (Caltag Laboratories). DNA was labeled with 0.2 µg/ml DAPI.

EGFP-histone H1.2 vector

Human histone gene 1.2 was amplified from genomic DNA by PCR using the following primer pairs (hHis1.2-Forward 5'-GGATCCATGTCCGAGACTGCTCCTGCC-3' and hHis1.2-Reverse 5'-CCCGGGCTATTTCTTCTTGGGCGCCGC-3'). The underlined sequences denote the built in Bam HI and Sma I restriction sites for the forward and reverse primers respectively. Histone 1.2 was cloned into pCR2.1 TOPO vector (Invitrogen) and Histone 1.2 inserts (654 bp) were isolated by Bam HI/Sma I digestion. These fragments were cloned into Bgl II/Sma I digested vector set pAcGFP1-C1 to develop in-frame EGFP-Histone 1.2 fusion genes, where the histone genes were fused to the C-terminal of enhanced green fluorescent protein so as to retain full functionality of the histone gene.

Expression of GFP was monitored in real time using IX81 inverted microscope (Olympus), equipped with fluorescence and a cooled CCD camera (Orca, Hamamatsu). Images were acquired using GFP filter and processed using SlideBook (Olympus). A pure population of Histone 1.2-EGFP expressing cells was isolated by fluorescence activated cell sorting (FACS) (Fig. 2.1-b) (Umass medical school).

a. b.



c.

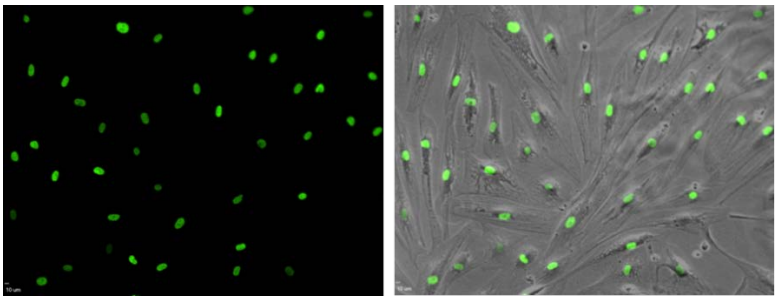


Figure 2.1 | **Generation of Histone H1.2 – EGFP expressing cell line**
(a) Human histone gene Histone 1.2 was amplified by PCR. The histone1.2 fragment was isolated and cloned downstream of an EGFP vector pAcGFP1-C1, C2 and C3 to develop C-terminal fusions. (b) Upon cell transfection cells were sorted using FACS and (c) pAc-GFP1-C1-Histone 1.1/1.2 transfected cells showed nuclear localization

***Xenopus laevis* egg extract preparation**

Metaphase arrested egg extracts were prepared using published protocols (Danilchick et al., 1991). Mature *Xenopus laevis* females were superovulated with pregnant mare's serum gonadotropin (PMSG, Calbiochem) and 72 hours later induced to ovulate with human chorionic gonadotropin (hCG, Calbiochem). Eggs were collected in cold MMR buffer: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM Hepes and washed 2 times with High Salt Barth Solution (NaCl 110 mM, Tris-HCl 15 mM, KCl 2 mM, $NaHCO_3$ 2 mM, $MgSO_4$ 1 mM, Na_2HPO_4 0.5 mM, EGTA 2 mM). The jelly coats were removed

with cold 2% L-cystein free base with 2 mM EGTA at pH 7.8 (adjusted with 6N NaOH). Eggs were washed in inactivating extract buffer (50 mM KCl, 50 mM Hepes, 5 mM $MgCl_2$, 5 mM EGTA, 2 mM β-mercaptoethanol). Eggs were crushed by centrifugation at 10,000 rpm for 15 minutes. The cytoplasmic layer was removed and centrifuged at 20,000 rpm for 15 min at 4°C. Translucent layer was removed and diluted 1:2 with extract dilution buffer at 4°C (50 mM KCl, 50 mM Hepes, 0.4 mM $MgCl_2$, 0.4 mM EGTA) supplemented just before use with 2 mM DTT, 10 µg/ml aprotinin, leupeptin and cytochalasin B, each. Extracts were centrifuged again at 100,000 x g for 1.5 hours at 4°C. High-speed egg extract was aliquoted at 50 µl/vial, snap frozen, and stored in liquid nitrogen. Total RNA was isolated and used for electroporation to determine whether RNA components alone could account for the fraction activity.

Extract fractionation by gel filtration chromatography

High-speed egg extract in extract dilution buffer was supplemented just before use with 2mM DTT, 10 µg/ml each aprotinin, leupeptin and cytochalasin B. The material was processed over Superdex200 gel filtration resin (GE Healthcare) using an XK26/100 column (GE Healthcare). Superdex200 (S200) resin was equilibrated with extract dilution buffer overnight at 4°C and the column was calibrated by running gel filtration standards (BioRad Laboratories). Ten percent column volume of high speed extract was loaded using an AKTÄ Explorer A200 FPLC and flow rate was maintained constant at 2 ml/min. During the run, absorbance was recorded at A280nm and A254nm. The chromatogram generated from the fractionation process was analyzed in comparison with the chromatogram generated from the column calibration. A total of ninety-two 4 ml fractions were collected in the range between 10 and 600 kDa. Concentration of protein and RNA in each fraction was determined by fluorometry (Qubit, Invitrogen). The proteins in each 4 ml fraction were acetone precipitated and resuspended to 10 mg/ml final concentration in extract dilution buffer. Samples for denaturing SDS-PAGE analysis were prepared by mixing 40 µl of each fraction with 10 µl of 5X SDS-PAGE loading buffer.

Gels underwent electrophoresis at 160 V for 1 hour (BioRad Laboratories) and silver stained. RNA was isolated from fractions using the procedure described below and DNA was removed by treatment with rDNase (according to the manufacturer's instructions; Ambion) and separated on 4% agarose gels.

Extract electroporation

Approximately $1x10^6$ cells were electroporated in 100 µl of pooled extract fractions (500 µg/ml protein) with three pulses (10 seconds apart), at 0.56 kV/cm for 1 msec/pulse (BTX). Electroporated cells were resuspended in serum-free medium. Removal of EGFP-Histone H1 was monitored in real time using Olympus IX81 inverted microscope equipped with environmental chamber. Images were acquired using Slidebook software (Olympus) during the 2-hour period post electroporation. Cells were subsequently plated in DMEM/F12, 10% FCIII, with or without 4 ng/ml FGF2. All cell cultures were carried out at 37°C, 5% O_2, 5% CO_2 and high humidity.

RNA extraction and RT-PCR

Total RNA was isolated using Trizol (Invitrogen) following the manufacturer's protocol. Four µg of total RNA was used to perform first strand cDNA synthesis using Superscript (Invitrogen). PCR was performed using 0.5 µl of first strand cDNA in Mg^{2+} free PCR TaKara polymerase buffer supplemented with 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 25 pmoles each of forward and reverse primers and 0.5U of TaKara ExTaq polymerase per reaction. PCR cycling was done as follows: Initial denaturation at 95°C for 2 minutes followed by 35 cycles of denaturation at 95°C for 15 seconds; annealing at primer-specific annealing temperature for 1 minute; and extension at 72°C for 1:30 minutes. Final extension was done at 72°C for 10 minutes and the samples held at 4°C until use. Amplification products were resolved on 2% agarose gels containing 0.5 µg/ml ethidium bromide in 1X TAE buffer and photographed using a Kodak 4000MM Image Station. Primers for amplification of human OCT4 (POU5F1) were designed to

detect the embryonic stem cell-specific transcript that produces the 360 amino acid variant (NM_002701). Primer sequences used in this study are presented in Table 1.

Table 2.1 | **Primer sequences used for stem cell gene amplification**

Target Gene	Forward primer	Reverse Primer	Amplicon size (bp)	Genomic size (bp)	Accession Number
Oct4	5'-GTTGATCCTCGGACCTGGCTA-3'	5'-GGTTGCCTCTCACTCGGTTCT-3'	646	5339	NM_002701
Nanog	5'-TGTCTTCTGCTGAGATGCCTCACA-3'	5'-CCTTCTGCGTCACACCATTGCTAT-3'	387	4811	NM_024865
hTert	5'-GCTTCCTCAGGAACACCAAGA-3'	5'-TGCAACTTGCTCCAGACACTC-3'	298	1657	NM_198253
Gapdh	5'-ATCACCATCTTCCAGGAGCGA-3'	5'-TTCTCCATGGTGGTGAAGACG-3'	101	101	NM_002046

Western Blotting

High speed-cleared cytoplasmic egg extract and cell lysate was supplemented with complete protease inhibitor cocktail (PIC, Santa Cruz Biotechnology) and 1 mM DTT. Protein concentration was determined with Quant-iT protein assay kit (Invitrogen). Equal amounts of protein and 2X sample buffer (BioRad Laboratories) were mixed and heated to 95°C for 5 minutes. Proteins were separated on 4-20% gradient SDS-PAGE gels and transferred to nitrocellulose membranes (BioRad Laboratories) using Towbin's transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol and 0.037% SDS). The membranes were blocked with Tween-Tris buffered saline (TBST: 25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 0.2% Tween), 5% dry milk (Santa Cruz Biotechnology) and 5% FCS. The same buffer, supplemented with 0.2% Tween was used for primary and secondary antibody incubations. Primary antibodies against OCT4 (Chemicon), NANOG (Abcam), FGF2, TGFβ-1 and maskin (all from Santa Cruz Biotechnology), and nucleoplasmin (Developmental Studies Hybridoma Bank) were used. Appropriate HRP-conjugated secondary antibodies were used for detection. In between antibody incubations, membranes were

washed three times with TBST. Membranes were incubated in luminol (Santa Cruz Biotechnology) and luminescence detected with Kodak 4000MM imager.

Immunocytochemistry

Cells were fixed with ice-cold methanol or 2% methanol-free formaldehyde. Samples were treated with 1 N HCl prior to blocking and labeling. Cells were blocked with 5% fetal calf serum (FCS) in Tween (0.2%)-Tris buffered saline (TBST). Primary antibodies against and fibroblast specific protein - FSP (Sigma), NANOG (Abcam), OCT4 (Chemicon), nestin (Abcam), and neuronal β-tubulin III (Tuj1, Covance) were diluted in TTBS and 3% FCS. Cells were labeled for 45 minutes at room temperature with primary antibodies. Plates were washed three times with TTBS and appropriate secondary antibody conjugated to AlexaFluor 488 (Molecular Probes) diluted in TTBS with serum added for 45 minutes. Antibodies were crosslinked with 4% formaldehyde for 5 minutes, and washed three times with DAPI added to the last wash. Plates were stored in TBS in the dark at 4°C until imaged. Cells were analyzed using IX81 inverted microscope (Olympus), equipped with automatic stage (Prior), fluorescence and a cooled CCD camera (Orca, Hamamatsu). Images were acquired using appropriate filters and processed using SlideBook (Olympus).

Differentiation protocols

Adipogenic differentiation

Cells were cultured in growth medium supplemented with 10 µM dexamethasone, 100 mg/ml 3-isobutyl-1- methylxanthine, 50 mM indomethacin and 10 mg/ml insulin (all from Calbiochem) for 3 weeks with a medium change twice a week. Cells were then rinsed twice with phosphate-buffered saline, fixed with 10% formalin for 10 min, washed with distilled water, rinsed in 60% isopropanol and covered with a 0.3% oil red O solution (HyClone) in 60% isopropanol. After 10 min, cultures were briefly rinsed in 60% isopropanol and thoroughly in distilled water and dried at room temperature.

Osteogenic differentiation

Cells were grown in medium supplemented with 1 µM dexamethasone, 10 mM β-glycerophosphate disodium and 50 mg/ml ascorbic acid (all from Sigma). After 4 weeks, cells were rinsed twice with PBS, fixed with formalin for 10 min, and washed with distilled water. To stain calcium deposits, cells were covered with a 2% aqueous solution of alizarin red S (Sigma) at pH 4.2, for 3 min. Cultures were then washed thoroughly with distilled water and dried at room temperature.

Neuronal differentiation

Cell aggregates were transferred to cell culture dishes and cultured in Neurobasal medium with 1X N2 (Invitrogen), 4 ng/ml FGF2 and 1X insulin, selenium and transferrin (ITS, Invitrogen) for 10 days. Neuroepithelium-like cultures were immunostained with antibodies against nestin and neuronal β-tubulin III and detected with AlexaFluor 488 and AlexaFluor 568-conjugated secondary antibodies, respectively. DNA was stained with DAPI.

Teratoma formation assay

Animal studies were done with IACUC approved protocols and in accordance with animal care and use procedures at Worcester Polytechnic Institute, Worcester, MA. One million of control and one million of extract treated fibroblasts were mixed with 8–12 µm diameter carbon beads in sterile Dubelcco's Phosphate Buffered Saline (DPBS) and injected into the hind leg muscle of SCID mice (Charles River Laboratories, Wilmington, MA). Animals were euthanized 6 weeks after injection, the muscle excised, and processed for histology. Tissues were fixed in 4% formaldehyde in DPBS and embedded in paraffin. Sections were stained with H&E and the injection site located by microscopic visualization of the carbon beads.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by

the Institutional Animal Care and Use Committee of the Worcester Polytechnic Institute (Protocol Numbers: #07-05 and 08-010). All efforts were made to minimize animal pain and suffering.

Role of exogenously added FGF2 in reprogramming

Role of exogenously added FGF2 in reprogramming of human dermal fibroblasts by *Xenopus laevis* egg extract fractions was tested through blocking of FGF receptor kinase activity using the chemical inhibitor SU5402 (Sigma-Aldrich, St. Louis MO), depletion of FGF2 from the total *Xenopus* extract and through removal of exogenously added FGF2 from the culture media after electroporation. After electroporation with total *Xenopus* egg extract, 100μM of inhibitor was added to the cells and incubated under conditions described above. Cells were analyzed by immunocytochemistry (ICC) at day 2 after treatment for activation of ERK signaling pathway through monitoring of ERK phosphorylation with phosphor-specific anti-ERK antibody. Total *Xenopus* egg extract was also depleted of FGF2 by immunoprecipitation with FGF2 specific antibody conjugated on agarose beads. Reprogramming activity of imunodepleted extract was tested in comparison with total *Xenopus* egg extract. Lastly, role of exogenously added FGF2 in reprogramming of human dermal fibroblasts by *Xenopus* egg extract fractions was tested by removing exogenously added FGF2 from the culture media after electroporation. Reprogramming effect under both conditions was tested and compared through monitoring expression of stem cell specific proteins in reprogrammed cells.

RESULTS

Cell morphology and *in vitro* life span

To eliminate the variability of cell responses due to the stage of the cell cycle, human dermal fibroblast cells were synchronized in G_1 by gradually reducing serum concentration in the culture medium over 10 days from 10% to 0.5% (Figure 2.2 A-a). A BrdU assay was performed to show that all

the cells had stopped cycling, thereby synchronizing them in G_0. By the end of the serum reduction period, cells had stopped proliferating, as indicated by the absence of BrdU incorporation over 24 hours of culture (Figure 2.2 A-b). Upon electroporation in the presence of *Xenopus laevis* egg extract and addition of serum to the culture medium, both extract treated cells as well as control cells re-entered the cell cycle. (Figure 2.2 A-c). To verify that the electroporation protocol indeed enabled transfer of large protein molecules into cells, we supplemented the extract with goat AMCA-conjugated IgG (Abcam) and monitored fluorescence in cells over 2 hours post-electroporation (Figure 2.2 B-a). The morphology of electroporated cells gradually changed during the following 6 weeks in culture, and cells displayed higher nucleus to cytoplasm ratio. Cells were grown as adherent monolayers (Figure 2.2 B-b). For differentiation experiments cells were transferred to low adhesion tissue culture plastic and allowed to form aggregates resembling embryonic bodies (Figure 2.2 B-c).

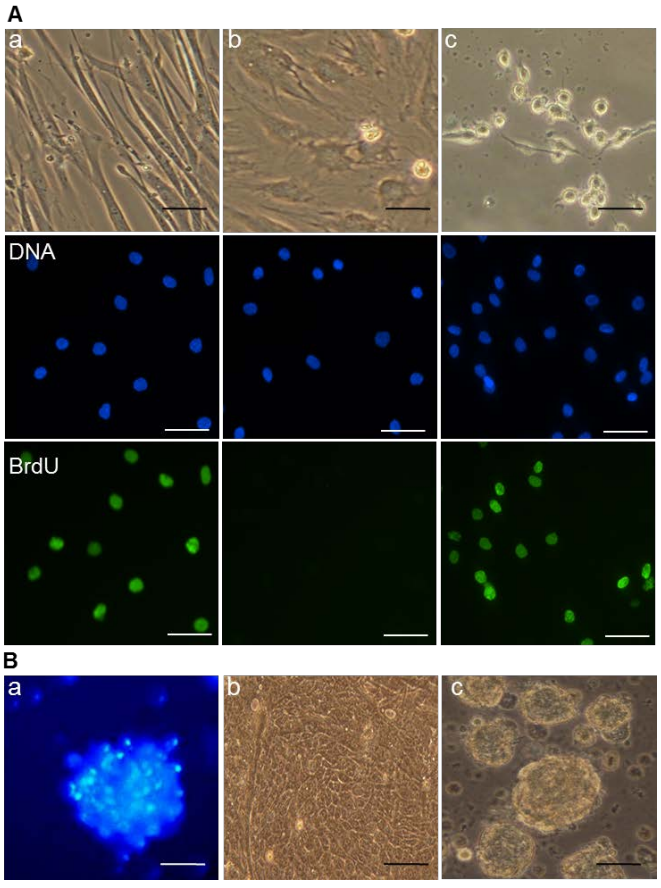


Figure 2.2 | **Morphology, cell cycle synchronization, uptake of *Xenopus* egg extract, and long term culture of electroporated human fibroblasts**. Human dermal fibroblasts were grown to subconfluence (A, column a). Serum concentration was gradually reduced over 10 days to drive cells into G1 as shown by lack of BrdU incorporation (A, column b). After electroporation (A, column c). (B) Uptake of extract was verified by ACMA-labeled IgG fluorescence (a) and cells were grown either in adherent monolayers (b) or embryonic bodies (c). Bars 20 um (A) and 100 um (B).

To examine whether or not the new cell phenotype could be maintained for prolonged periods of time, we cultured extract-treated and untreated cells in DMEM/F12, 10% FCIII (Fetal Clone III, Hyclone) with or without exogenously supplemented FGF2 (4 ng/ml, PeproTech). The number of population doublings between the two was significantly different depending on the presence or absence of FGF2. FGF2 supplemented cultures underwent over 60 population doublings as compared to 25-35 population doublings for non-FGF2 cultures (Figure 2.3).

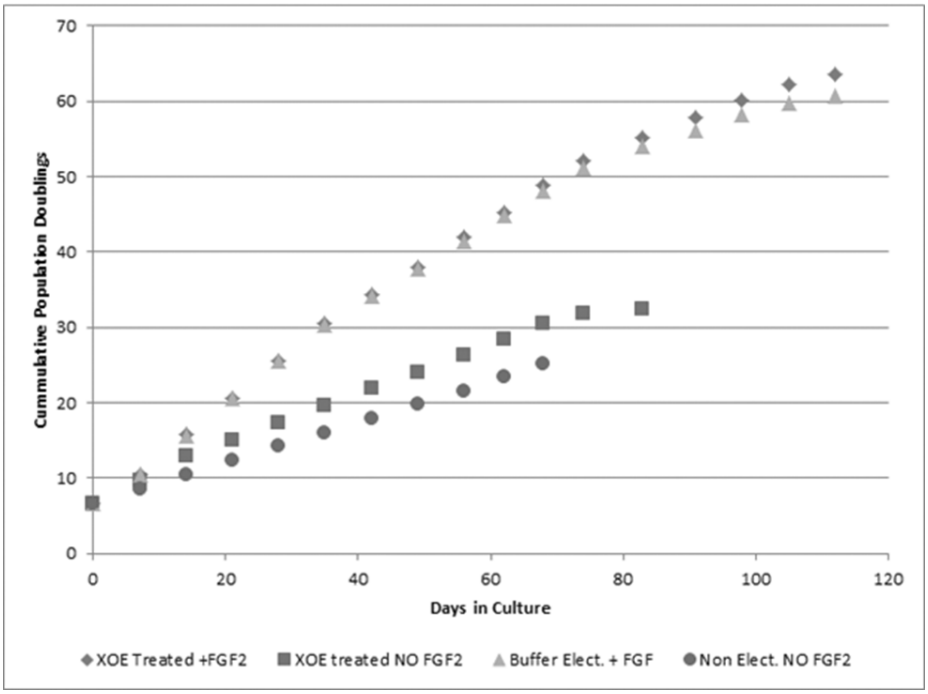


Figure 2.3 | **Cumulative population doubling data**
Population doubling data of human dermal fibroblast electroporated with *Xenopus* egg extract (XOE treated) and supplemented with FGF2 (diamond), electroporated with *Xenopus* egg extract (XOE treated) and no FGF2 added (squares), buffer electroporated control treated with added FGF2 (triangles), and non-electroporated human dermal fibroblast control grown with no added FGF2 in the culture media (circles).

Removal of histone H1.2 - EGFP

Removal of over 90% of linker histones during the initial stages of reprogramming has been previously reported in somatic nuclei incubated with *Xenopus* egg extract (Dimitrov and Wolffe 1996).

To ascertain that the *Xenopus laevis* egg extract affected the initial stages of chromatin remodeling in fibroblast nuclei, we transfected human fibroblasts with EGFP-histone H1 fusion protein vector. Stable transfectants showed a high proportion of cells expressing nuclear localized EGFP-Histone H1 protein. Subsequent removal of nuclear fluorescence was monitored in real-time during 2 hours post-electroporation with *Xenopus* egg extract. The strength of the fluorescence signal was compared to non-electroporated cells and cells electroporated with buffer alone. Electroporation with complete *Xenopus laevis* egg extract induced the rapid removal of histone H1 (Figure 2.4 A) and the majority of the protein was undetectable 60 minutes after electroporation. Cells were subsequently electroporated with pools of ten adjacent extract fractions obtained from the Superdex200 column. Only pools of extract fractions that demonstrated histone H1 removal were used for the electroporation experiments on non-transformed fibroblasts.

Expression of early developmental genes

To evaluate stem cell transcription factor activation, we investigated the presence of developmentally important transcripts and their protein products in extract electroporated cells. No effect of extract electroporation on transcription of OCT4 or NANOG was observed on day 7 after electroporation (Figure 2.4b). Non-electroporated fibroblasts supplemented with FGF2 in the culture medium contained mRNA for all the transcription factors examined, an observation that we have recently extended for several stem cell specific genes in human dermal fibroblasts treated with FGF2 under low oxygen conditions (Page et al., 2009). There was no detectable telomerase hTERT transcript in control fibroblasts, and electroporation alone or with extract fractions did not appear to induce hTERT expression. This increase in population doublings was accompanied by the maintenance of their stem cell transcription factor expression and the absence of hTERT (Figure 2.4b).

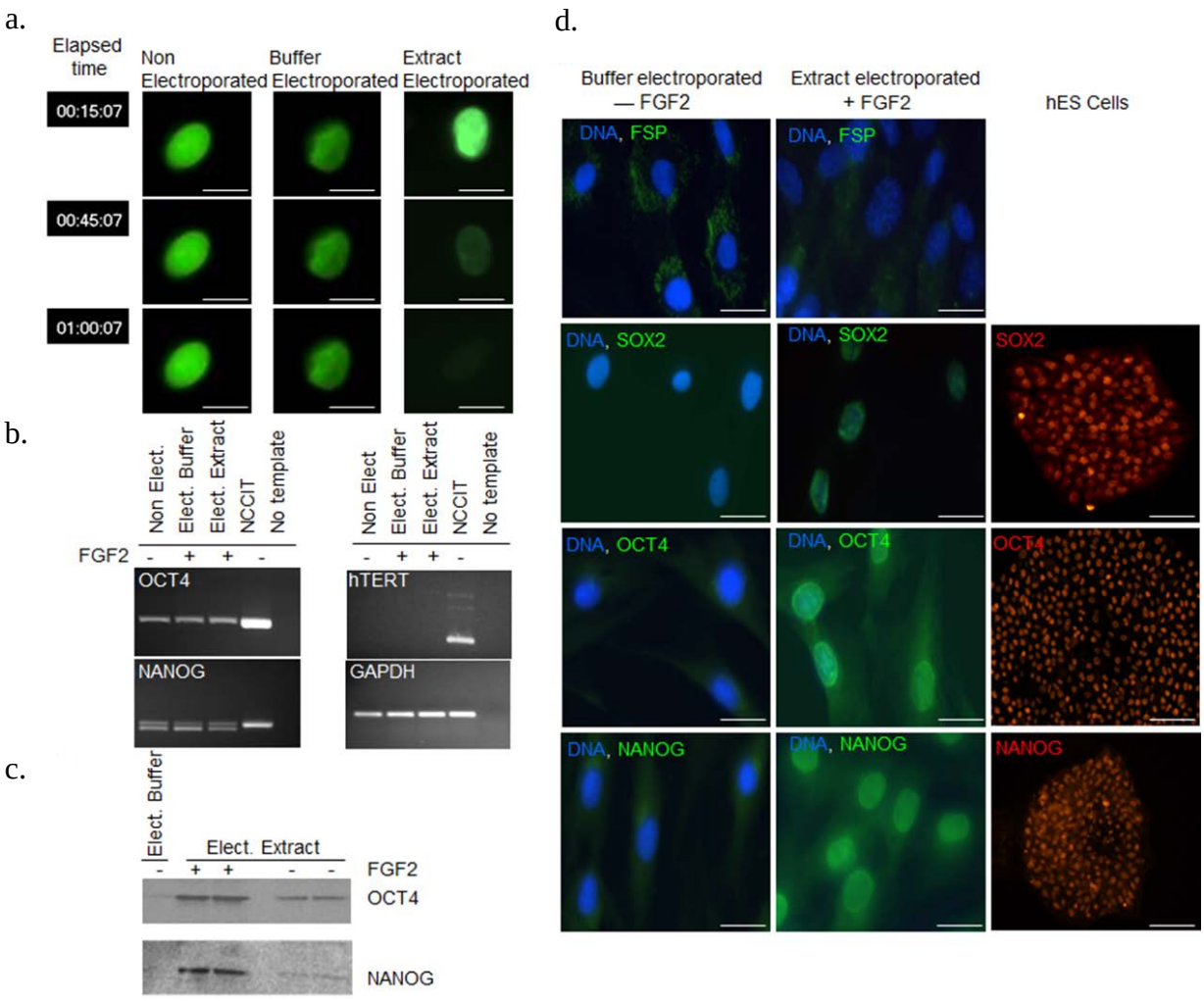


Figure 2.4 | **Removal of histone H1 and expression of stem cell genes in electroporated human fibroblasts** (a) Time-lapse of EGFP-histone H1 fusion protein removal from adult fibroblast nuclei at 15, 45 and 100 minutes after extract delivery. (b) RT-PCR analysis. Cells were not electroporated (Non Elect.), electroporated with extract dilution buffer (Elect. Buffer) or with *Xenopus* egg extract (Elect. Extract). Teratocarcinoma cells (NCCIT) were used as a positive control and No template as a negative control. FGF2 was supplemented in the culture media as indicated by (+) or (-). (c) Western blot analysis for presence of OCT4 and NANOG in cells electroporated with extract dilution buffer (Elect. Buffer) and extract electroporated cells (Elect. Extract) grown with or without FGF2 for 7 days. (d) Immunocytochemistry of extract electroporated and buffer electroporated fibroblasts for fibroblasts specific protein (FSP), SOX2, OCT4, and NANOG. Green – Alexafluor 488 conjugated secondary antibody; Red – Alexafluor 568 conjugated secondary antibody; Blue – Dapi. Bars 10 µm (A and D-Buffer and Extract treated cells); bars 200 µm (D-hES Cells).

Examination of cells using Western blotting and immunocytochemistry (ICC), however, demonstrated that levels of OCT4 and NANOG increased only in the extract electroporated group, indicating translational activation of these genes (Figure 2.4c and 2.4d). In parallel with the induction of OCT4 and

NANOG mRNA translation, was a concomitant reduction in the levels of fibroblast specific protein (FSP) expression (Figure 2.4d). OCT4 and NANOG proteins localized to the nuclei of extract electroporated cells, which would correspond to their site of activity as transcription factors. Overall, approximately 30% of the extract electroporated cells displayed the above described protein expression and localization properties compared to none of the control electroporated cells grown without exogenously added FGF2. When tested alone for its potential to contribute to expression of stem cell genes, FGF2 had the ability to induce the expression of OCT4 and NANOG proteins, as observed previously (Page et al., 2009; Page et al., 2011), an effect that was also achieved by the extract in the absence of exogenous FGF2 (Figure 2.4c).

Components of *Xenopus laevis* egg extract

Our strategies in identifying active "dedifferentiation activity" components employed an extract fractionation approach, reconstitution of activity with combinations of pools of adjacent fractions, and an evaluation of the activity by a 2-step bioassay. Step 1 involved real-time monitoring of the removal of nuclear histone H1, and Step 2 involved the detection of OCT4 and NANOG proteins. Based on this 2-step bioassay, two distinct pools of fractions were identified after Superdex200 fractionation. The high molecular weight pool (~150-200 kDa) contained 10 fractions, and the low molecular weight group (~15-40 kDa) contained 20 fractions. A combination of the high and low molecular weight pools together with addition of exogenous FGF2, reconstituted the activity of total extract (Figure 2.5a, Figure 2.6 a-d).

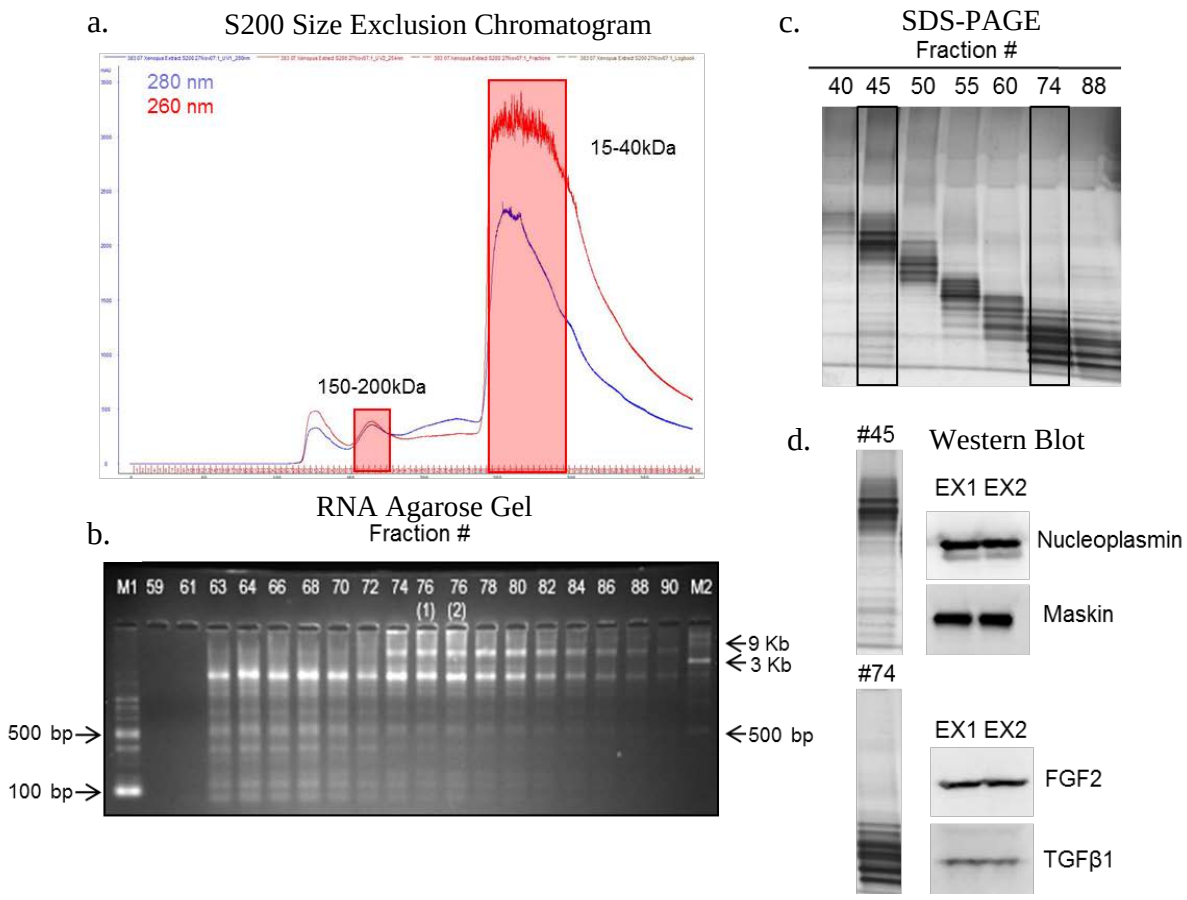


Figure 2.5 |**Superdex 200 fractionation of *Xenopus laevis* egg extract and screening for reprogramming factors**
Peak alignment from extract fractionation; red boxes outline groups of fractions with reprogramming activity (a). Agarose gel separation of RNAs (b) and silver stained SDS-PAGE analysis of some of the fractions collected during the fractionation step (c). Western blotting detected nucleoplasmin, maskin, FGF2, and TGFb1 in high and low molecular weight fractions, respectively (d). EX1 and EX2 represent protein from two independently isolated egg extracts

Interestingly, the electroporation of extract RNA alone did not induce the removal of histone H1 or the induction of OCT4/NANOG protein expression, but instead caused significant cell death.

RNA was isolated from approximately every other fraction between fractions 28 and 92. Four percent agarose failed to detect any RNA up to fraction 58-61, depending on the run (Figure 2.5b).

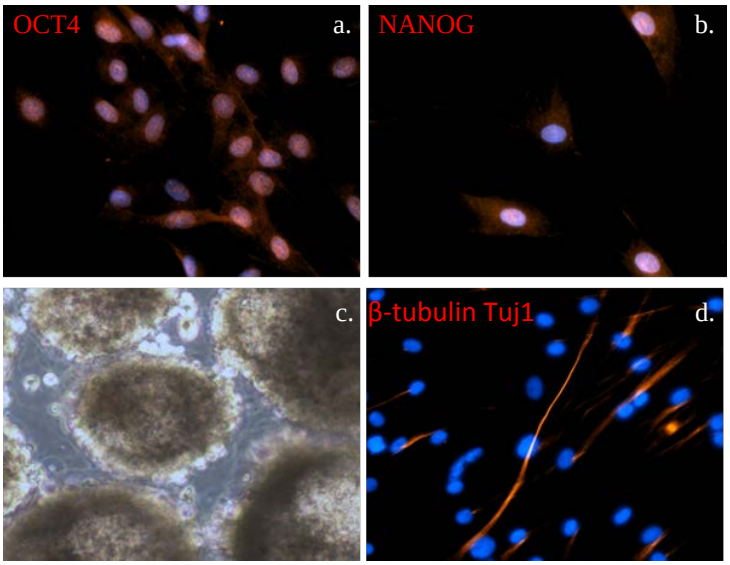


Figure 2.6 | ***In vitro* differentiation of human dermal fibroblasts treated with select *Xenopus laevis* egg extract pools.**
Treated cells express nuclear OCT4 (a), and Nuclear NANOG protein (b). Electroporated cells can grow as embryonic bodies (c), and supported maturation of cells into neurons (d - neuronal specific β-tubulin Tuj1)

Similar RNA profiles were observed in fractions 63 through72, and a different pattern was observed in fractions 74 through 90, with very little trace of RNA remaining in fraction 90. The lower number fractions exhibited the presence of distinct bands of small RNAs; their levels gradually decreased in the higher fraction. These small RNAs were above 100 bases long or more, suggesting that they may include either primary miRNA or pre-miRNA (Ambady et al., 2012). The pools including fractions 45 and 74 that contain activity to induce OCT4/NANOG protein expression (Figure 2.6 a, b) also contained significant amounts of large RNAs that were not detectable in fractions without this activity (Figure 2.5b, lanes 59 - 72). Silver staining of SDS-PAGE separated protein indicates significant separation by size of proteins in various fractions, and the proteins eluted at the expected molecular weight ranges (Figure 2.5c). Western blot analyses identified the presence of FGF2 and TGF-β1 in the low molecular weight fractions (I.e. Fraction 74) and nucleoplasmin and maskin in the high molecular weight fractions (I.e. Fraction 45 (Figure 2.5d).

Differentiation potential of extract-electroporated cells

Extract treated human dermal fibroblast cells were grown in aggregates resembling embryonic bodies (Figure 2.7a). In three independent replicates, fibroblasts used for de-differentiation at 10, 15

and 20 population doublings after treatment repeatedly followed the expected differentiation pathway. The potential of cells to follow an ectodermal tissue lineage development was indicated by acquisition of a neuroepithelial phenotype (Figure 2.7b) and expression of nestin and Tuj1 (Figure 2.7c and d), both are markers of neuronal differentiation. The deposition of calcium in electroporated cells exposed to osteogenic conditions was detected with Alizarin Red (Figure 2.7e). Under conditions that promote adipogenic differentiation, cells began accumulating lipids, as detected with oil red-O stain (Figure 2.7f). Control (non-electroporated) cells remained fibroblast-like and showed no presence of adipogenic cells, Ca^{2+} deposits or expression of neuronal markers. When injected into SCID mice, the extract treated, FGF2 grown cells did not form teratomas.

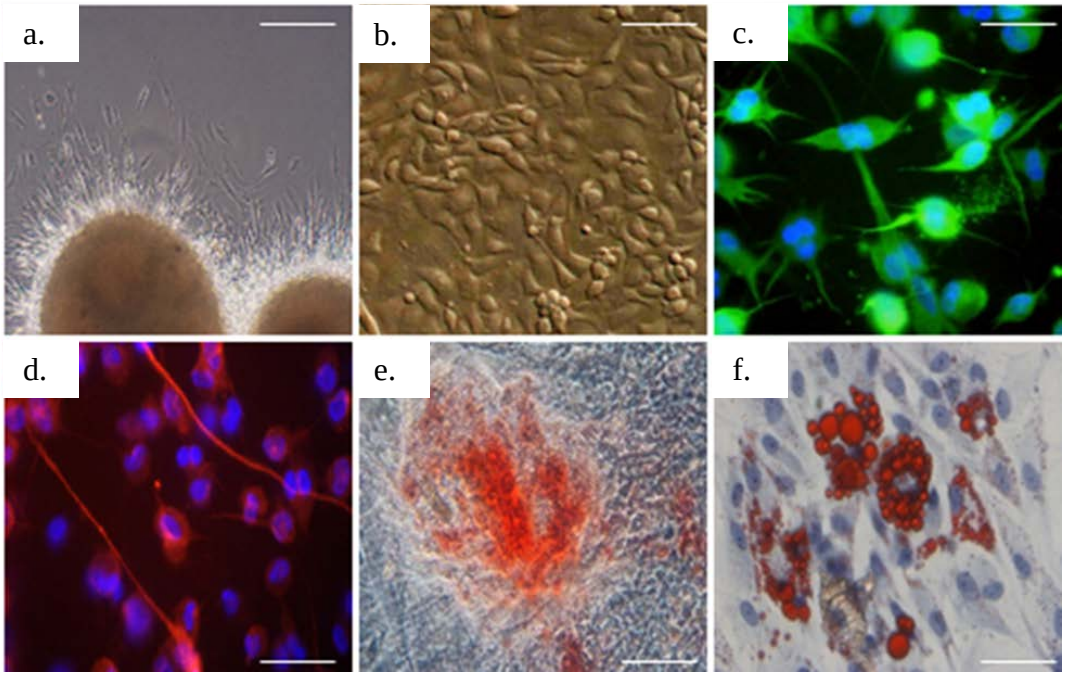


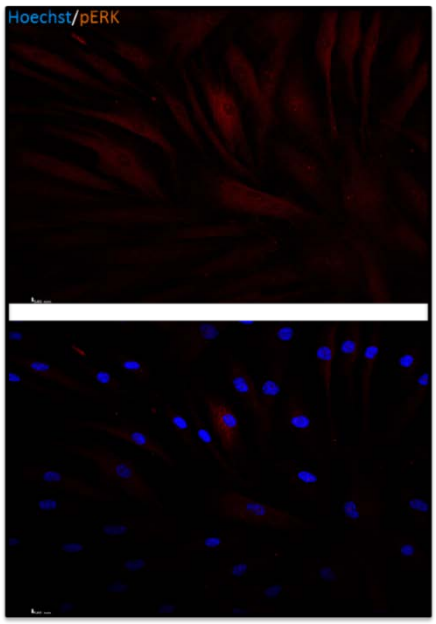
Figure 2.7 |***In vitro* differentiation of extract-treated fibroblasts.** Embryonic-like bodies (a) formed neuroepithelial-like cells (b), expressed nestin (c) and supported maturation of cells into neurons (d - neuronal specific β-tubulin Tuj1). Osteogenic induction produced cells expressing osteocalcin, **detected with Alizarin Red** (e), adipogenic induction produced lipid-accumulating cells **detected with oil red O stain and counterstained with hematoxylin** (f). Bar 200 µm (a), and 50 µm (b-f).

Role of exogenously added FGF2 in maintenance of multipotency in reprogrammed cells

The addition of FGF receptor inhibitor SU5402 to extract electroporated cells resulted in an almost complete inhibition of the ERK pathway as demonstrated by lack of ERK phosphorylation in inhibitor treated cultures (Figure 2.8a). Thus, the FGFR appears to be important for ERK activation in human dermal fibroblast cells

a.

CRL2352 DMEM / F12 + 10% FCIII + FGF2 (18kDa) + **SU5402**



b.

CRL2352 DMEM / F12 + 10% FCIII + FGF2 (18kDa)

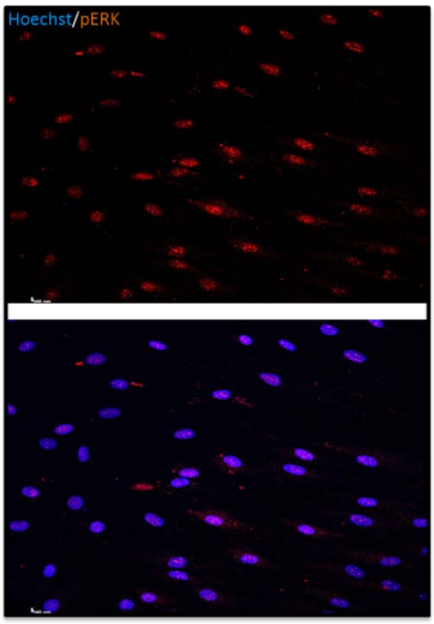
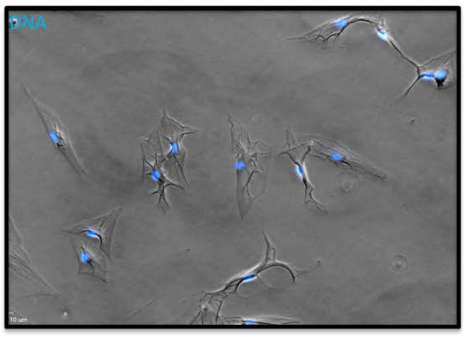


Figure 2.8 | **Short term effect of SU5402 FGF receptor inhibitor on Xenopus egg extract treated cells** (a) Cells treated with SU5402 for 2 days after electroporation and (b) cells not treated with inhibitor but grown under normal reprogramming growth conditions

Culturing of extract treated cells under the presence of SU5402 inhibitor for 8 days resulted in lower proliferation rates, and phenotypical analysis of the inhibitor treated cells demonstrates that these cells are stressed and undergoing cell death (Figure 2.9a). To eliminate the possibility that cell death was a result of cytotoxicity and not ERK1/2 pathway inhibition, we also tested reprogramming potential of FGF2 depleted *Xenopus* cytoplasmic extract.

a.

CRL2352 DMEM / F12 + 10% FCIII
+ FGF2 (18kDa) + **SU5402**



b.

CRL2352 DMEM / F12 + 10% FCIII
+ FGF2 (18kDa)

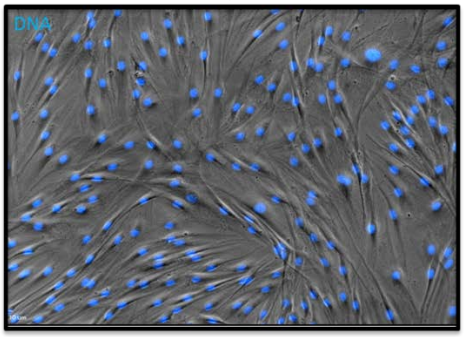


Figure 2.9 | **Long term effect of SU5402 FGF receptor inhibitor on Xenopus egg extract treated cells** (a) Cells treated with SU5402 for 8 days after electroporation and (b) cells grown under normal conditions

Electroporation of human dermal fibroblasts with total *Xenopus* egg extract or FGF2-depleted extract did not show any differences during the early reprogramming events; both conditions demonstrated the loss of nuclear Histone H1.2-GFP after electroporation. However, addition of exogenously added FGF2 in the culture media was necessary for sustaining the expression of stem cell specific proteins in electroporated cells (Figure 2.10).

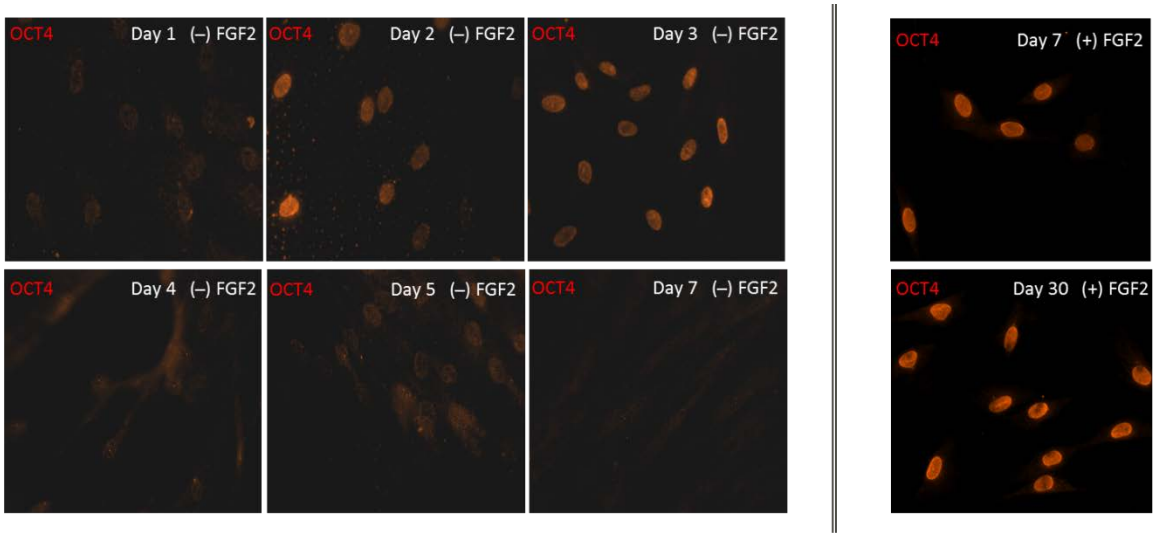


Figure 2.10 | **Role of exogenously added FGF2 in maintaining expression of stem cell specific proteins** Sustained OCT4 expression in *Xenopus* extract treated human dermal fibroblasts depends on the duration of exposure to FGF2 and requires the presence of exogenously added FGF2. Alexafluor-568 conjugated secondary antibody (red).

Discussion

In order to reprogram a differentiated cell into a multi- or pluripotent phenotype, a number of molecular changes must take place. It has been proposed that a sequence of events including the remodeling of nuclear chromatin leading to the selective activation or repression of gene transcription is required for transient or stable alteration of cell fate. Removal of DNA binding proteins, such as linker histone H1 proteins is important for initial chromatin decondensation (Kikyo et al., 2000), and is also important for the acquisition of transcriptional pluripotency - a process that is efficiently accomplished by nucleoplasmin (Dimitrov and Wolffe 1996). The use of EGFP-histone H1 fusion protein allowed monitoring of its removal from the nucleus upon exposure to extract fractions. Our observation of rapid disappearance of EGFP-histone H1 is indicative of at least a partial erasure of the cells' epigenetic status, and is similar in its kinetics to the one observed by Dimitrov and Wolffe (Dimitrov and Wolffe 1996). Two pools of extract fractions were identified that in combination and with addition of exogenous FGF2 demonstrated reprogramming activity and contained nucleoplasmin, a known H1 chaperone.

The removal of histone H1 can result in the transcriptional activation of specific genes such as 5S rRNA (Bouvet et al., 1994), and is followed by chromatin decondensation and the establishment of transcriptionally permissive chromatin. RT-PCR analysis of transcripts for several early developmental and stem cell associated genes, however, did not reveal a significant change in the levels of any of the transcripts examined (Figure 2.4b). This confirms our recent data showing that a variety of differentiated cell lines contained detectable amounts of both *OCT4* and *NANOG* transcripts (Ambady et al., 2010). Low levels of *OCT4* expression have previously been reported in leukocytes (Hansis et al., 2004), peripheral blood mononuclear cells (Zangrossi et al., 2007), and recently in adult human fibroblasts (Page et al., 2009). The levels of NANOG and OCT4 transcripts, however, have no direct predictive value for the levels of their respective proteins. It has been shown that the levels of transcripts represent both the embryonic gene and its pseudogenes, with which they share significant homology (Ambady et al., 2010;

Atlasi et al., 2008; Zangrossi et al., 2007)**.** While little or no effect of egg extracts in our study can be ascribed to transcription of the select stem cell genes, the translation of their mRNAs was significantly affected by the extract in combination with exogenously added FGF2, and this potential mechanism needs further investigation.

Immunocytochemistry and Western blotting detected the presence of OCT4 and NANOG proteins in treated cells, and these transcription factors localized to the cells' nuclei. We previously described a very similar post-transcriptional activation of these genes using defined culture conditions with FGF2 supplementation of the cell culture medium (Page et al., 2009), however, exogenously supplemented FGF2 alone does not have the potential to de-differentiate human dermal fibroblasts into a multipotent state. Our active extract fraction pools contain significant amounts of FGF2 based on Western blot analysis, similar to previously reported amounts in complete *Xenopus* egg extract (Kimelman et al., 1988). Our findings show that full reprogramming of human dermal fibroblasts through permeabilization *in vitro* requires both the combination of active fraction pools from the extract plus exogenously added FGF2. While FGF2 has not been implicated previously in transcriptional activation of *OCT4* or *NANOG*, it has been determined that the maintenance of expression of these genes and cell pluripotency is dependent on FGF2 (Levenstein et al., 2006). The proposed action of FGF2 involves the induction of members of the TGF-β pathway; TGF-β ligands maintain the expression of OCT4, SOX2, and NANOG which in turn activate the expression of endogenous FGF2 that completes this regulatory loop (Greber et al., 2007). It is reasonable to hypothesize that a similar FGF2-dependent core regulatory circuit maintaining the self-renewal ability and pluripotent state of embryonic stem cells may at least in part be needed for reprogramming of differentiated somatic cells. However, the lack of hTERT reactivation and lack of the potential of the cells to form tumors *in vivo* suggest that additional events may need to take place within the cells before true pluripotency is achieved. One of these missing

events could be regulated by extract RNAs, as only fractions containing, both protein and RNAs demonstrated reprogramming activity.

The widespread occurrence of protein/small RNA co-elution may indicate a novel mechanism of transcriptional, translational and/or post-translational regulation. Recently, Awe and Byrne (2013) identified eight candidate oocyte reprogramming factors (CORFs) that closely correlate with the "chromatin loosening/enhanced reprogramming" hypothesis previously described by Gurdon and Wilmut (2011). Five of those eight CORFs (ARID2, ASF1A, ASF1B, H1FOO and KDM6B) not only are expressed in *Xenopus laevis* oocytes (Awe and Byrne 2013), but their molecular weights fit the S200 elution profile in our study, suggesting that these factors may be present in the pools of fractions that can reconstitute the reprogramming activity of the total extract. Small RNAs, microRNAs in particular, are becoming increasingly important in our understanding of posttranscriptional regulation. While hundreds of targets will likely emerge from this ongoing research, it may be possible to identify a much smaller number of the "absolutely required" targets through the development of an integrated map of components, their relationships, and their established cellular activities. This approach would enable the selection of the most upstream acting molecules, their testing in the bioassay, and progressive elimination of unlikely targets. It is also expected that a number of novel proteins will be identified that may play an important role in the process.

The natural reprogramming activity of egg cytoplasm without the need for induced transcription may lead to the identification of the molecules and mechanisms of nuclear reprogramming, and consequently the use these molecules to enhance the efficiency of reprogramming could be applied to the derivation of therapeutically relevant, patient-specific cell types. This would be of interest especially if the reprogrammed cells of one type could yield cells of another unrelated, therapeutically important cell-type, rather than being pluripotent and potentially tumorigenic.

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Chapter 3 *Role of FGF2 isoforms in nuclear reprogramming and induction of stem cell specific genes and proteins*

Abstract

Basic fibroblast growth factor (FGF2) is a member of a large family of growth factors known to have broad mitogenic and angiogenic activities. Five isoforms of human FGF2 result from the initiation of translation at four different CUG and one AUG start codons (Arese, Chen et al. 1999, Arnaud, Touriol et al. 1999). The four CUG initiated isoforms (34kDa, 24kDa, 22.5kDa and 22kDa the high molecular weight isoforms (HMW)) are predominantly nuclear, while the AUG initiated isoform (18kDa) or low molecular weight isoform (LMW) is predominantly secreted and acts through FGF receptor tyrosine kinases. FGF2 is involved in many processes including the expansion and survival of pluripotent human embryonic stem cells, and is one of the key factors responsible for maintaining the balance between survival, proliferation and self-renewal. HMW FGF2 isoforms have also been described as capable of being released from cells through extracellular vesicle shedding and the vesicles have the potential to stimulate plasminogen activator production and chemotaxis (Taverna, Ghersi et al. 2003). Here we report the over-expression and isolation of highly pure recombinant FGF2 isoforms, and demonstrate their biological activity in comparison with the well described 18kDa isoform when added exogenously to the culture medium of primary human dermal fibroblasts. We also show that HMW FGF2 isoforms have the ability to support pluripotency and the self-renewal of human embryonic stem cells grown on a mouse embryonic feeder (MEF) layer or a feeder-free system. Furthermore, we demonstrate that treatment with exogenously supplemented FGF2 isoforms in the culture media increases population doubling numbers, shortens population doubling time and induces the expression of stem cell specific proteins in primary human dermal fibroblasts.

Introduction

FGF2 is a member of the FGF family known as heparin binding factors and was first purified as a heparin-binding molecule from bovine pituitary (Gospodarowicz, Cheng et al. 1984). FGFs demonstrate

their biological activity through the activation of surface FGF receptors (FGFR) (Ornitz, Xu et al. 1996, Zhang, Ibrahimi et al. 2006), and possess broad mitogenic and angiogenic activity. Five isoforms of FGF2, ranging from 18kDa to 34kDa exists as a result of translation initiation at alternative start sites (Florkiewicz and Sommer 1989, Arnaud, Touriol et al. 1999). HMW FGF2 isoforms, 22kDa, 22.5kDa 24kDa and 34kDa respectively, are co-linear extensions of the 18kDa isoform (Florkiewicz and Sommer 1989, Arnaud, Touriol et al. 1999). The HMW isoforms are predominantly intracellular and localize to the nucleus, while the 18kDa isoform is mainly cytoplasmic and is secreted from cells by a process that is not fully understood. The localization of FGF2 isoforms varies depending on the cellular conditions and stimuli (Arese, Chen et al. 1999), and HMW isoforms have been described in some occasions to be released in the extracellular space through a process known as vesicle shedding (Taverna, Ghersi et al. 2003). Human dermal fibroblasts express all five isoforms of FGF2 and specific FGF receptors (Prindull, Prindull et al. 1978, Lee, Johnson et al. 1989, Dellera, Presta et al. 1991, Root and Shipley 2000, Bryant, Wylie et al. 2005, Eiselleova, Matulka et al. 2009). FGFR activation results in the stimulation of various signal transduction cascades that have been implicated in multiple aspects of vertebrate and invertebrate embryonic development, tumor growth, angiogenesis, wound healing, and physiology (Martin 1981, Hughes 1997, Tachibana, Amato et al. 2013). Miss-expression of FGFs has also been shown to be associated with several cancers (Becker, Mc et al. 1963, Hattori, Odagiri et al. 1990, Yayon, Klagsbrun et al. 1991). The LMW (18kDa) isoform is the best studied, and is reported to have intracrine, autocrine as well as paracrine effects (Arese, Chen et al. 1999, Reilly, Mizukoshi et al. 2004). Binding of the FGF2 ligand to the receptor triggers receptor dimerization, phosphorylation of its kinase domain, and activation of several intracellular kinase pathways which result in ERK1/2 phosphorylation. Investigating the ability of each isoform to bind the receptors and activate downstream kinase pathways and identifying FGF-FGFR binding specificities is critical to understanding the biological mechanisms involved in normal development and pathogenesis. Previous research has demonstrated that HMW

FGF2 isoforms can be transported to the extracellular environment (Presta, Gualandris et al. 1993) and the 24kDa FGF2 isoform administered exogenously to endothelial cells induces cell proliferation with a similar dose response as the 18kDa isoform (Taverna, Ghersi et al. 2003). Previous publications have identified FGF2 as an important factor in maintaining pluripotency in human stem cells (Eiselleova, Matulka et al. 2009, Zoumaro-Djayoon, Ding et al. 2011). Our lab has shown that the 18kDa FGF2 isoform, in combination with culturing in 5% O_2, induces the expression of stem cell specific genes and proteins in human dermal fibroblasts (Page, Ambady et al. 2009).

The multifunctional nature and large variety of effects makes FGF2 an important target for study, and for these reasons the generation of highly pure, active protein is essential. Heparin chromatography has been used for purification of 18kDa FGF2 isoform, however, heparin contamination in purified FGF2 preparations has been previously described to interfere with the stability and biological activity of FGF2 (Eiselleova, Matulka et al. 2009, Gasparian, Elistratov et al. 2009). To avoid heparin contamination and achieve high purity after a single chromatography step, in this study FGF2 isoforms were synthesized as fusion proteins containing a 6xHis tag. The 6xHis tagged FGF2 isoforms demonstrated high affinity for Ni-NTA, and their biological activity was compared to commercially available 18kDa FGF2 (Peprotech, Rocky Hill, NJ). We investigated the ability of HMW FGF2 isoforms to bind and phosphorylate FGF receptor 1 and activate the mitogenic pathway through ERK1/2 phosphorylation. We also show the potential of HMW FGF2 isoforms to support growth of hES cells in an undifferentiated state and induce the expression of stem cell specific proteins in adult human dermal fibroblasts. The findings of this study show that all HMW FGF2 isoforms supplemented exogenously in the culture media of adult human dermal fibroblasts can increase population doubling number as well as shorten population doubling time in human dermal fibroblasts, with similar activity as the 18kDa isoform.

Materials and Methods

Design and synthesis of FGF2 isoforms

All FGF2 isoforms are translated from the same mRNA through translation initiating at alternative translation start sites. Alternative translation start sites in the recombinant FGF2 sequences were mutated to ensure exclusive expression of a single isoform from each construct (Fig. 3.1a). Each construct was designed as a 6xHis-tag fusion protein with a tobacco etch virus (TEV) recognition site between the 6xHis-tag and the protein coding sequence (Fig. 3.1b).

a. CTGGTGGGTGTGGGGGGTGGAGATGTAGAAGATGTGACGCCGCGGCCCGGCGGGTGCCAGATTAGCGGACGCGGTGC
CCGCGGTTGCAACGGGATCCCGGGCGCTGCAGCTTGGGAGGCGGCTCTCCCCAGGCGGCGTCCGCGGAGACACCCATCC
GTGAACCCCAGGTCCCGGGCCGCCGGCTCGCCGCGCACCAGGGGCCGGCGGACAGAAGAGCGGCCGAGCGGCTCGAG
GCTGGGGGACCGCGGGCGCGGCCGCGCGCTGCCGGGCGGGAGGCTGGGGGGCCGGGGCCGGGGCCGTGCCCCGGAG
CGGGTCGGAGGCCGGGGCCGGGGCCGGGGGACGGCGGCTCCCCGCGCGGCTCCAGCGGCTCGGGGATCCCGGCCGGG
CCCCGCAGGGACCATGGCAGCCGGGAGCATCACCACGCTGCCCGCCTTGCCCGAGGATGGCGGCAGCGGCGCCTTCCCG
CCCGGCCACTTCAAGGACCCCAAGCGGCTGTACTGCAAAAACGGGGGCTTCTTCCTGCGCATCCACCCCGACGGCCGAGT
TGACGGGGTCCGGGAGAAGAGCGACCCTCACATCAAGCTACAACTTCAAGCAGAAGAGAGAGGAGTTGTGTCTATCAAA
GGAGTGTGTGCTAACCGTTACCTGGCTATGAAGGAAGATGGAAGATTACTGGCTTCTAAATGTGTTACGGATGAGTGTTT
CTTTTTTGAACGATTGGAATCTAATAACTACAATACTTACCGGTCAAGGAAATACACCAGTTGGTATGTGGCACTGAAACG
AACTGGGCAGTATAAACTTGGATCCAAAACAGGACCTGGGCAGAAAGCTATACTTTTTCTTCCAATGTCTGCTAAGAGCT
GA

b.

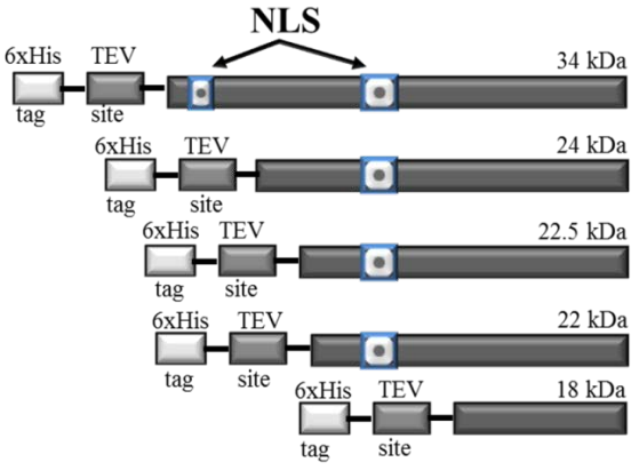


Figure 3.1 | **Recombinant FGF2 isoform design** Mutation of alternative translation start sites (a), Design of FGF2 isoforms with a 6xHistidine tag and a TEV cleavage site (b)

NheI and EcoRI restriction sites were included on the NH_2-terminal end, and a HindIII restriction site was included on the COOH-terminal end to simplify sub-cloning in other expression vectors. Constructs for all five isoforms were synthesized by Epoch Life Sciences (Texas Medical Center) in a pBluescript (+)

cloning vector. DNA Inserts for all five FGF2 isoforms were isolated by EcoRI and HindIII (New England Biolabs) restriction digest and purified by agarose gel electrophoresis. Each FGF2 isoform was subcloned in pFastBac1 (Life Technologies) vector using the same restriction sites, allowing for directional insertion of the gene of interest. The correct orientation of the insert was confirmed by restriction digest.

Human FGF2 isoform over-expression

Bac-to-Bac® baculovirus expression system (Life Technologies) was used to overexpress FGF2 isoforms in SF21 cells, and Infection Kinetics Monitoring (IKM®) (Blue Sky Services Inc.) was used to monitor transfection efficiency and infection rates. pFastBac® I vectors containing individual constructs for each FGF2 isoform were transformed in DH10Bac competent cells (Life Technologies). Recombinant bacmid DNA was isolated using manufacturer's protocol and 40mL cultures of SF21 cells were transfected with each isoform using Cellfectin® II reagent (Life Technologies), following manufacturer's protocol. P1 virus was harvested at 72-80 hours after transfection when cell viability was between 60-70%. Clarified supernatant containing the P1 viral stock for each FGF2 isoform was used to test expression in an 18 point small-scale expression optimization experiment. Twenty-four hours prior to infection SF9 and SF21 cultures were seeded at 7.5 x 10^5 cells/ml in SF900 II SFM media (Life Technologies). A pilot expression study in 50 ml cultures was set up for infection for each viral dilution 1:100/1:1000/1:10,000, in both SF9 and SF21 cell lines (Table 3.1). Recombinant protein expression was analyzed by Western blot analysis at 24, 48 and 72 hours after infection. The best expression conditions were selected based on Western blot analysis of expression optimization results (Appendix A). Two 500ml cultures in 1L flasks were infected for each construct.

Table 3.1 | **18-point small-scale expression optimization in insect cell expression system**

Construct	Cell line	Harvest time post-infection	Virus dilutions
34kDa	SF9	24h 48h 72h	1:100 1:1,000 1:10,000
	SF21		
24kDa	SF9	24h 48h 72h	1:100 1:1,000 1:10,000
	SF21		
22.5kDa	SF9	24h 48h 72h	1:100 1:1,000 1:10,000
	SF21		
22kDa	SF9	24h 48h 72h	1:100 1:1,000 1:10,000
	SF21		
18kDa	SF9	24h 48h 72h	1:100 1:1,000 1:10,000
	SF21		

Cells were seeded at $7.5x10^5$ cells/ml and 24 hours later infected with P1 virus stock at a 1:1000 concentration. Cells were harvested by centrifugation 72 hours post infection, flash frozen in liquid nitrogen and stored at -80^{o}C. Cells from a 500µl aliquot were analyzed by Western blot analysis to confirm recombinant protein expression for all five individual FGF2 isoforms.

Recombinant Human FGF2 Isoform Purification

Cells from 1L expression were subjected to purification over Ni-NTA resin. Cells were lysed in 10ml ice cold lysis buffer (50mM Tris pH 7.5, 250mM NaCl, 1mM DTT, EDTA free protease inhibitor cocktail) per 1 gram of cell paste using a Missonix XL-2000 ultrasonic cell disruptor, for 3 x 40 pulses at power 15. Clarified soluble fraction was generated by centrifugation at 12,000 x g for 2 hours at 4^{o}C. A 5.0 ml Ni-NTA column (Qiagen) was equilibrated with 10 column volumes of lysis buffer. Clarified soluble

fraction was loaded over the column using an AKTA Explorer FPLC (GE Healthcare) at a 5ml/min flow rate. The column was washed to baseline with lysis buffer. A second wash to baseline was performed with lysis buffer containing 30mM imidazole. Protein was eluted with lysis buffer containing 300mM imidazole and 2.5ml fractions were collected and analyzed by coomassie stained SDS-PAGE. Ni-NTA resin was stripped with lysis buffer containing 1M imidazole. All starting material and fractions collected were analyzed by coomassie stained SDS-PAGE and Western blot analysis with anti-His antibody (Appendix B). Fractions containing greater than 90% pure protein by coomassie stained SDS-PAGE were pooled together and dialyzed in 1x PBS overnight. The dialyzed pool was centrifuged at 20,000 x g for 1 hour and then filtered through a 0.2µm filter. Final protein concentration was determined using Bradford protein assay (Thermo Scientific) using BSA as a standard. The final material was analyzed by coomassie stained SDS-PAGE gel and Western blot using monoclonal mouse anti-His antibody (Santa Cruz Biotechnology).

RECOMBINANT HUMAN FGF2 ISOFORM ACTIVITY

Receptor phosphorylation and mitogenic pathway activation

Primary adult human dermal fibroblasts (CRL #2352, American Tissue Culture Collection) at passage p8 were plated in 6-well tissue culture plates (Thermo Scientific) and in 12-well plates on coverslips, in DMEM:Ham's F12 (50:50), supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific) and 4 mM L-glutamine (Mediatech). Cultures were grown to 80% confluence using standard procedures. On the day of the assay, the growth media was replaced with serum-free media, 2 hours prior to treatment. At the time of the assay, complete growth media was replaced with serum free DMEM:Ham's F12 (50:50) media containing human recombinant FGF2 isoforms at a final molar concentration of 222.22 ± 0.4pM. Serum free DMEM:Ham's F12 (50:50) containing commercially available 18kDa human FGF2 (Peprotech) was used as a positive control. Serum free DMEM:Ham's F12

(50:50) with no supplements was used as a negative control. All cultures were incubated for 30 minutes at 37°C, 5% O_2, 5% CO_2 and high humidity. At the end of the incubation period, cells were washed once in PBS. Coverslips were used for ICC analysis using the method described. Cells from 6-well plates were washed once in PBS, harvested in 2X Laemli buffer, lysed by sonication using a Missonix XL-2000 ultrasonic cell disruptor for 3 x 10 pulses at power 2 and subjected to Western blot analysis using the indicated antibodies.

Maintenance of hES Cell Pluripotency

Human embryonic stem cells (hESC) line WA09 (H9, WiCell) were cultured on in-house-derived mouse embryonic fibroblasts (MEF) plated at a density of $2.25e10^4/cm^2$ on plates coated for 60 minutes with 1% gelatin solution (MP Biologicals). MEFs were inactivated with 10 µg/ml mitomyocin-c (Sigma-Aldrich,) for 2 hours before plating on gelatin coated plates. hESC were cultured in Knockout DMEM (Life Technologies), supplemented with 15% Knockout serum (Life Technologies), 2.0mM Glutamax (Life Technologies), 50mM 2-Mercapptoethanol (Sigma-Aldrich), 1x MEM non-essential amino acids (Mediatech) and 8ng/mL basic FGF (Peprotech). Medium was replaced daily except for the first day after plating to allow hESCs to attach. Cells were passaged when colonies had grown for five days or become confluent. hESC were dissociated using non-enzymatic cell dissociation buffer (Life Technologies) and split 1:12 onto new MEFs feeder layer prepared as described above. OCT4, NANOG and LIN28 protein expression was analyzed by Western blot analysis with specific antibodies using the method described below.

Induction of stem cell protein expression and population doubling in human dermal fibroblasts

Primary adult human dermal fibroblasts from connective tissue isolated from tissue biopsy from a below-knee amputation of a 24-year-old male (CRL-2352) were obtained from American Tissue Culture

Collection (ATCC) at passage 2. Cells were cultured in medium consisting of DMEM:Ham's F12 (50:50, MediaTech) with 10% Fetalclone III (Hyclone). The DMEM (without L-Glutamine or phenol red) was supplemented with 4mM fresh L-Glutamine (MediaTech) prior to use. Cultures were incubated in a 37^{O}C incubator in a humidified environment of 5% CO_2, 5% O2. The number of population doublings was calculated as log2 (#final/#initial). Cells were seeded at 60,000 cells per well of a six well plate at each passage (Falcon). Human recombinant FGF2 isoforms (purified in our laboratory) and control 18kDa FGF2 (Peprotech) was supplemented into the medium at 4 ng/mL.

RT-PCR and RT-qPCR analysis

RNA from day 8 and day 18 hES cells and human dermal fibroblasts (CRL2352) cultured with different FGF2 isoforms, was extracted using TRIzol reagent (Life Technologies) according to the manufacturer's protocol, and quantified by spectrophotometry using a nanodrop 2000 (Thermo Scientific). One microgram of RNA was subjected to DNase digestion, followed by a reverse transcription using qScript™ cDNA Synthesis Kit (Quanta Biosciences), following manufacturer's protocol. RT-PCR was performed using GoTaq® green master mix (Promega) with 1 µl of DNA template, 0.5 µl forward primer (10 µM stock), 0.5 µl reverse primer (10 µM stock) in a 20 µl reaction. PCR cycling was done as follows: Initial denaturation at 95^{o}C for 7 minutes followed by 30 cycles of denaturation at 95^{o}C for 30 seconds; annealing at primer-specific annealing temperature for 1 minute; and extension at 72^{o}C for 20 seconds. The final extension was done at 72^{o}C for 7 minutes, and the samples held at 4^{o}C until use. Amplification products were resolved on 2% agarose gels containing 0.5 µg/ml ethidium bromide in 1X TAE buffer and imaged using a Bio Rad Gel Doc™ XR+ system (Bio Rad). Twenty ng/well of cDNA was used as a template in RT-qPCR reactions with intron-spanning primers specific for the genes of interest (Table 3.1). Non-template control and an RNA sample for each replicate were used to control for potential contamination. All RT-qPCR reactions were performed in triplicate and the resultant values were

combined into an average cycle threshold (Ct). Real-time SYBR green fluorescence (PerfeCTa SYBR Green FastMix w/ low ROX, Quanta Biosciences) was measured using a 7500 Real Time PCR system (Applied Biosystems). Expression profiles for the mRNA transcripts are shown as relative expression levels calculated by the ΔΔCt method as previously described (Schmittgen and Livak 2008). Normalization (ΔCt) was to actin. One way analysis of variance (ANOVA) and Tukey's post hoc test ($p<0.05$) were used to compare gene expression levels and determine if there were any statistically significant differences in gene expression. RT-PCR was performed using the same primers used for RT-qPCR for 30 cycles (Table 3.2).

Table 3.2 | **RT-qPCR primers for stem cell genes**

Target Gene	Forward primer	Reverse Primer	Amplicon size (bp)	Accession Number
Oct4	5'-TCGAGAACCGAGTGAGAGG -3'	5'-GAACCACACTCGGACCACA -3'	125	NM_001159542.1
Nanog	5'-ATGCCTCACACGGAGACTG -3'	5'-AAGTGGGTTGTTTGCCTTTG -3'	103	NM_024865.2
Lin28	5'-GAAGCGCAGATCAAAAGGAG -3'	5'-GCTGATGCTCTGGCAGAAGT -3'	115	NM_024674.4
Actin	5'-AGAGCTACGAGCTGCCTGAC-3'	5'-GGATGCCACAGGACTCCA-3'	111	NM_001101.3

SDS-PAGE and Immunoblotting

A 0.2 gram pellet from each transfected culture was lysed in 200µl cold lysis buffer (40mM Tris-HCl, 250mM NaCl, 8% glycerol, 0.0125% Brij-35, 0.01% Triton X-100, 0.005% Tween-20) with protease inhibitor cocktail. 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 Laemli sample buffer. A clarified soluble fraction was generated by centrifugation at 14,000 x g for 30 minutes at 4^{o}C, and mixed with 5X laemli sample buffer. Samples were boiled for 5 minutes and separated electrophoretically on 4-20% SDS-PAGE gel (Bio-Rad) using a Bio-Rad mini protean system. For SDS-PAGE analysis, the gel was stained with coomassie brilliant blue stain. For Western blot analysis, the proteins were transferred on PVDF membrane (Millipore) using a semi dry transfer apparatus (GE Healthcare). Membrane was blocked in TBST buffer (25mM Tris-HCl pH7.5, 130mM NaCl, 0.1% Tween-20) with 5% fat-free dry milk for 30

minutes. The membrane was incubated in indicated primary antibody overnight at 4^{o}C. After washing the membrane 3 x 10 minutes, the membrane was incubated in alkaline phosphatase-conjugated secondary antibody for 2 hours at room temperature. The bands were visualized using Western blue reagent (Promega). Images were acquired with a Canon LiDE 200 scanner.

Immunocytochemistry

Cells were fixed with ice-cold methanol or 2% methanol-free formaldehyde. Samples were permeabilized with 1 N HCl or 0.1% tritonX-100, respectively, prior to blocking and labeling. Cells were blocked with 5% bovine serum albumin (BSA) in TBST. Indicated primary antibodies were diluted in TBST and 1% BSA. Cells were labeled for 2 hours at room temperature. Plates were washed three times with TBST and appropriate secondary antibodies conjugated to AlexaFluor 568 diluted in TBST with 1% BSA added for 45 minutes. After being washed with PBS, the cells were incubated for 10 min at room temperature with rhodamine-phalloidin 488 (Life Technologies) to stain cellular F-actin. Coverslips were mounted using Prolong Gold® (Life Technologies) and analyzed using IX81 inverted microscope (Olympus), equipped with automatic stage (Prior), fluorescence and a cooled CCD camera (Orca Hamamatsu). Images were acquired using appropriate filters and processed using SlideBook (Olympus).

Results

Human FGF2 isoform over-expression

The initial efforts were focused on achieving high expression levels for all individual FGF2 isoforms. Over-expression in SF9 cells, using a 1:1000 viral dilution and harvested at 72 hours post infection were selected as best expression conditions based on the Western blot analysis of the 18-point small scale expression scout (Table 3.3 and Appendix A).

Table 3.3 | **Large scale (1L) expression conditions for FGF2 isoforms**

Construct	Cell line	Harvest time post-infection	Virus dilutions
34kDa	SF21	72h	1:10,000
24kDa	SF21	72h	1:10,000
22.5kDa	SF21	72h	1:10,000
22kDa	SF21	72h	1:10,000
18kDa	SF21	72h	1:10,000

The expression level and solubility of FGF2 isoforms isolated from 1L cultures was confirmed by Western blot analysis with anti-His antibody (Figure 3.2). The data show that bands of the expected molecular weight were present for all isoforms.

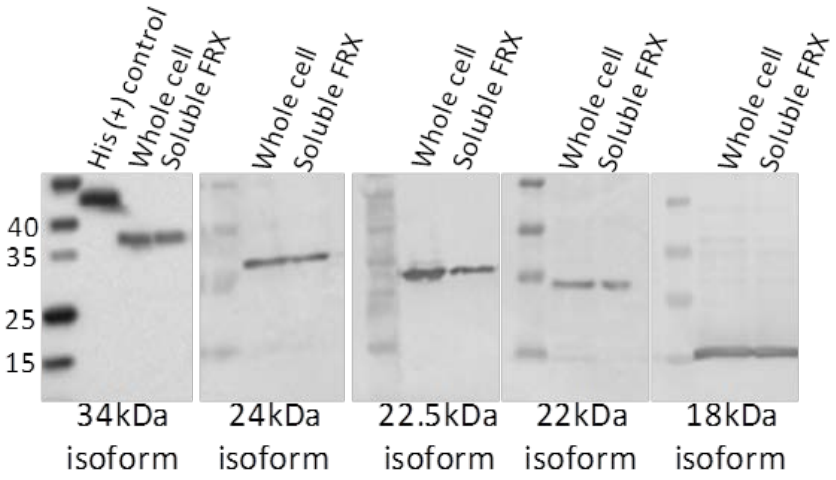


Figure 3.2 I **Commassie stained SDS-PAGE of overexpressed FGF2 isoforms** – human FGF2 isoform expression from 1L SF9 cultures infected with 1:1000 virus dilutions. His (+) control is a 6xhis tagged protein used to confirm specificity of the antibody. Whole cell lysate and soluble fractions were analyzed for each isoform.

Human Recombinant FGF2 Isoform purification

FGF2 isoforms were purified using metal affinity chromatography over Ni-NTA resin. Previous publications have shown heparin chromatography purification to interfere with biological activity of the

growth factor (Gasparian, Elistratov et al. 2009). One liter expression cultures of the baculovirus® expression system were purified using single step immobilized metal affinity chromatography (IMAC) over Ni-NTA resin (Qiagen). Only the fractions demonstrating over 85% purity by coomassie stained SDS-PAGE analysis (Appendix B) were pooled and dialyzed in phosphate buffered saline. Protein recovery was between 4mg – 10mg for different isoforms, with purity ranging from 85-90%, by coomassie stained SDS-PAGE gel analysis (Fig. 3.3a). However in some cases minor bands were observed which theoretically could affect the activity of the isoforms. Western blot analysis with anti-6xHis antibody confirmed identity of bands visible on coomassie stained SDS-PAGE (Fig. 3.3b).

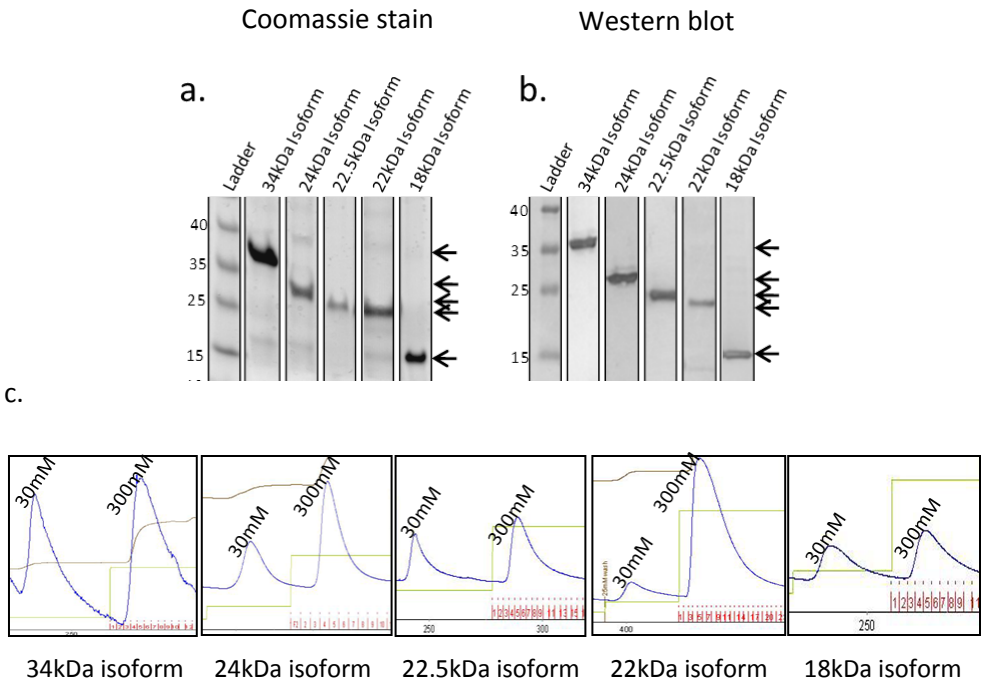


Figure 3.3 I **Purification of FGF2 isoforms** – Coomassie stained SDS-PAGE gel of final dialyzed material (a). Western blot analysis with mouse anti-His antibody (b). Chromatogram of recombinant human FGF2 isoforms purified over Ni-NTA column. 30mM peak corresponds to 30mM imidazole wash fraction. 300mM peak corresponds to elution fractions collected (c).

The chromatograph from the FPLC purification step showed clear peaks eluting with 300mM imidazole concentration (Fig. 3.3c). Final pools of FGF2 isoforms were dialyzed in phosphate buffered saline (PBS), and then diluted to 10µg/ml final concentration in buffer containing 1% BSA. Aliquots for each FGF2 isoform were flash frozen in liquid nitrogen and stored at -80^{O}C.

Recombinant Human FGF2 isoform activity

Previous studies have shown that binding of the 18kDa FGF2 ligand to the receptor tyrosine kinases induces receptor dimerization and phosphorylation followed by phosphorylation of fibroblast growth factor receptor substrate 2α (FRS2α). When testing the activity of our FGF2 isoforms, Western blot analysis with anti phospho-specific antibodies show that recombinant human FGF2 isoforms are able to phosphorylate FGF receptor 1 (Fig 3.4a), phosphorylate FRS2α (Fig. 3.4b), and activate the downstream ERK1/2 pathway (Fig. 3.4d), all with similar efficiency as the commercially available 18kDa isoform. The Western blot signals for each treatment were normalized to actin and were quantified using ImageJ software (Fig. 3.4 f,g,h). Western blot analysis with anti ERK1/2 antibody indicates that the expression level of total ERK1/2 protein is similar in the treated and untreated cultures (Fig. 3.4 e). Western blot analysis shows some baseline phosphorylation of FGFR1, FRS2α and ERK1/2 in the untreated human dermal fibroblasts. This might be due to endogenous FGF2 expression in human dermal fibroblasts.

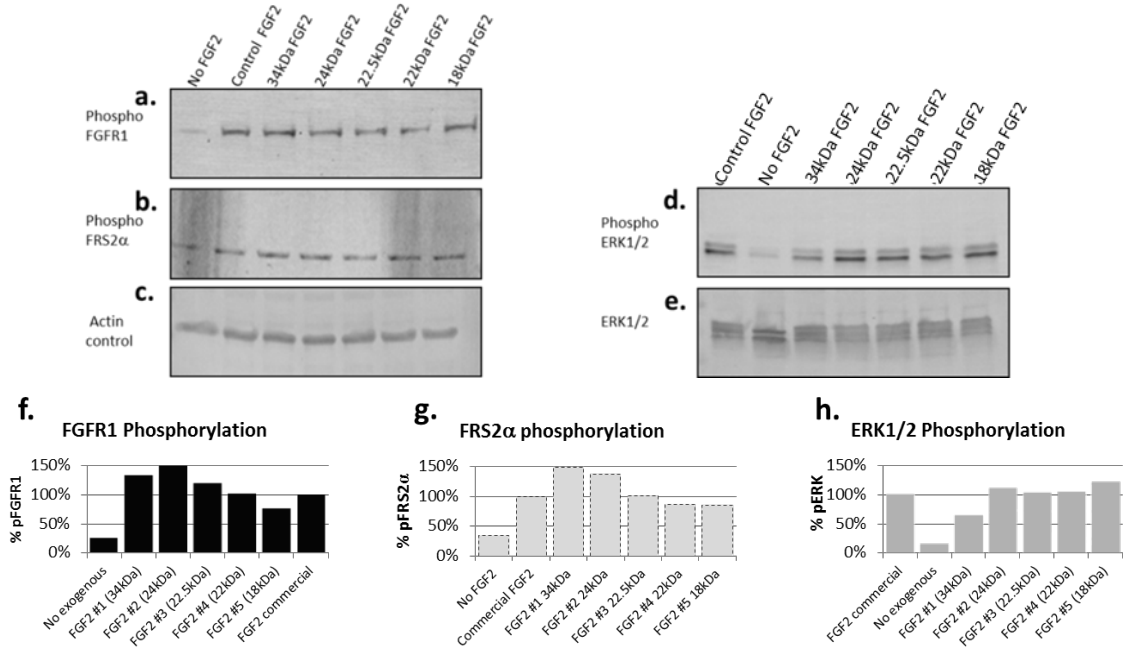


Figure 3.4 I **Recombinant human FGF2 activity** – Western blot analysis of CRL 2352 human dermal fibroblasts treated with recombinant human FGF2 isoforms; specific anti-phospho FGFR1 antibody (a); specific anti-phospho FRS2α (b); anti-actin antibody (c); anti-phospho ERK1/2 antibody (d); anti ERK1/2 antibody (e); Quantitation of Western blot signal using ImageJ analysis software (f; g; h)

Immunocytochemistry analysis with specific antibodies to phosphorylated ERK1/2 and phosphorylated FGFR1 shows the presence of phosphorylated Erk1/2 and phosphorylated FGF receptor 1 in all of the treated samples (Fig 3.5 a,b).

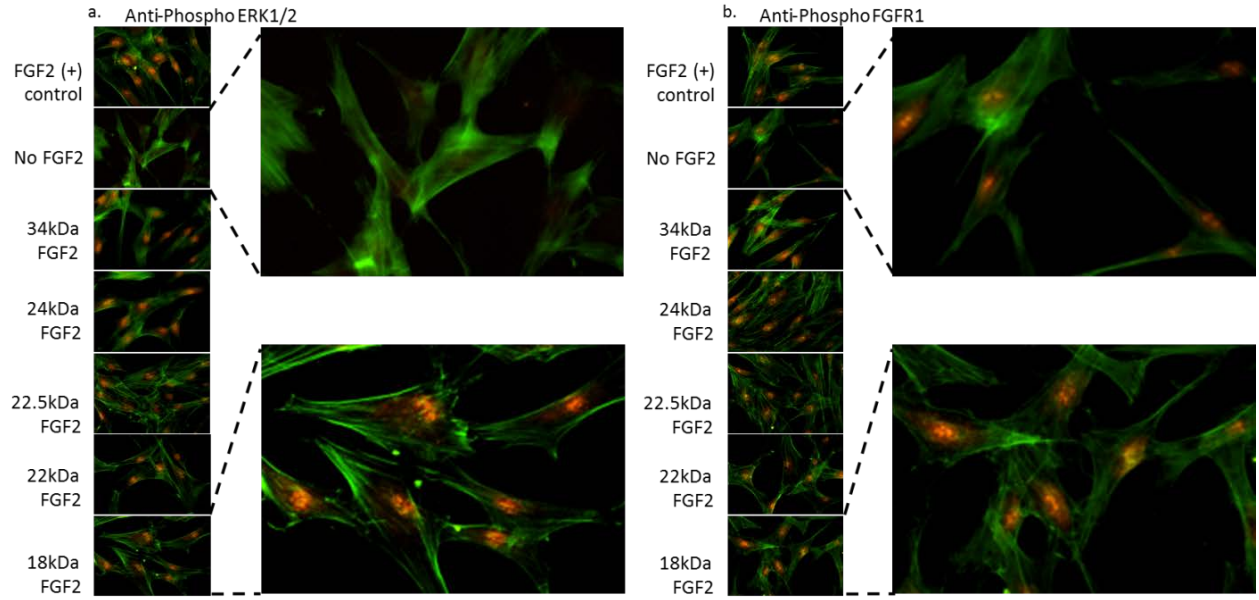


Figure 3.5 I **Recombinant human FGF2 activity** Immunocytochemistry analysis of CRL 2352 human dermal fibroblasts treated with recombinant human FGF2 isoforms; anti-phosphoERK1/2 antibody (a); specific anti-phospho FGFR1 antibody (b). Signal was visualized with alexafluor 568-conjugated secondary antibody (red). Filamentous actin was stained with Alexafluor 488-conjugated phalloidin (green).

FGF2 isoforms Support Pluripotency and Self Renewal of hES cells

Experiments investigating the ability of FGF2 isoforms to support growth of hES cells in an undifferentiated state and support the expression of stem cell specific genes and proteins demonstrated that all FGF2 isoforms have the ability to maintain hES cells in an undifferentiated state for a prolonged period of time. qRT-PCR and traditional RT-PCR analysis shows no significant difference in the gene expression levels between various FGF2 isoform treatments and the negative control at day 8 after treatment (Figure 3.6 a, b).

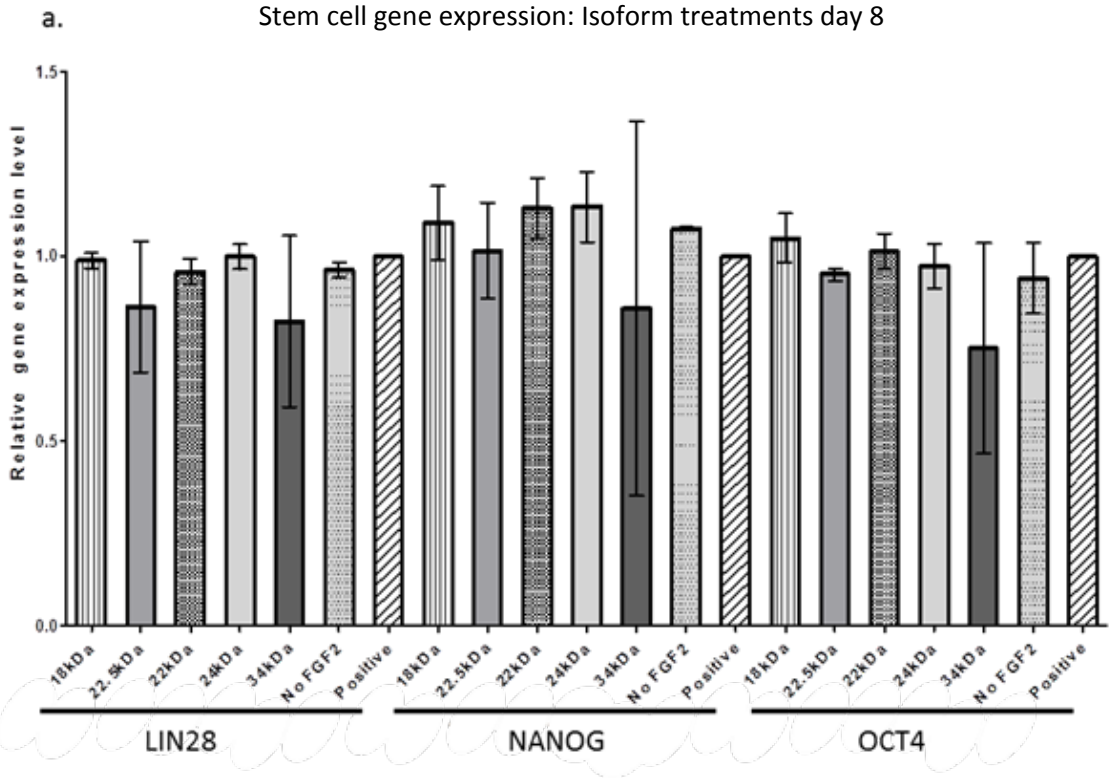


Figure 3.6 I **Day 8 RT-qPCR and traditional RT-PCR analysis** RT-qPCR analysis of day 8 treated hES cells with FGF2 isoforms. (a) and traditional RT-PCR analyzed on agarose gel (b). Commercially available FGF2 from Peprotech was used as a positive control. hES cell medium without exogenous FGF2 was used as a negative control. One way-ANOVA, Tukey's post hoc test at alpha=0.05 ($p>0.05$) was used to analyze RT-qPCR results. No statistical significance was detected between the isoform treatments and controls. Error bars indicate SD (n=2).

The results are similar for the expression of stem cell specific markers at day 8 after treatment, with all treatments showing no significant loss of protein expression. Immunocytochemistry and Western blot analysis show approximately equal expression of stem cell specific proteins in all treatments at day 8 (Figure 3.7 a, b, c).

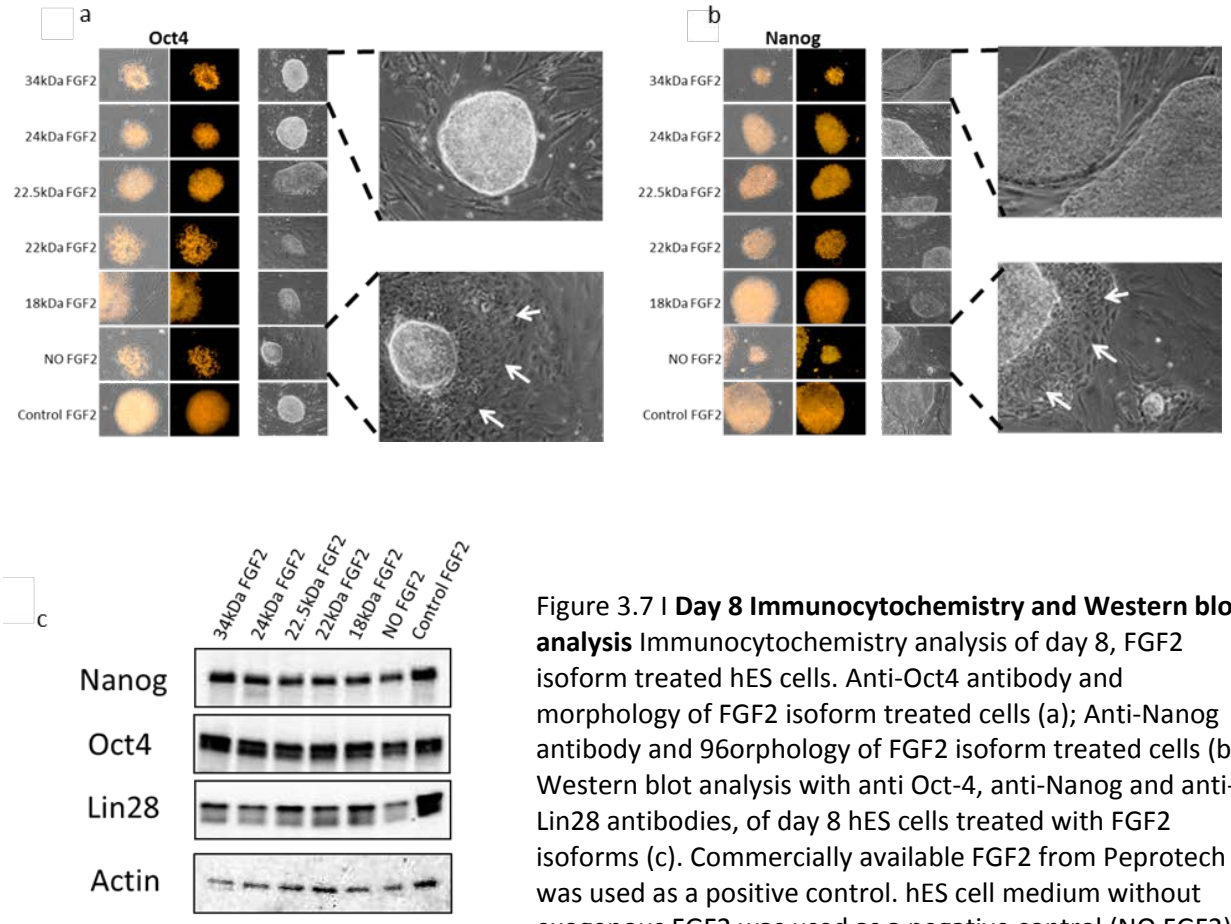
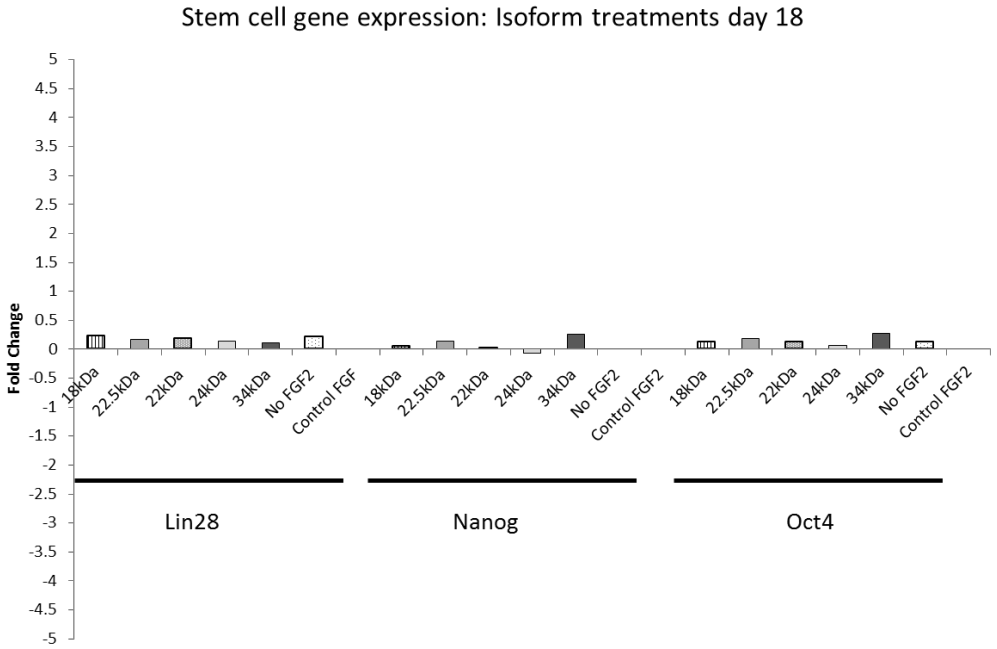


Figure 3.7 I **Day 8 Immunocytochemistry and Western blot analysis** Immunocytochemistry analysis of day 8, FGF2 isoform treated hES cells. Anti-Oct4 antibody and morphology of FGF2 isoform treated cells (a); Anti-Nanog antibody and 96orphology of FGF2 isoform treated cells (b); Western blot analysis with anti Oct-4, anti-Nanog and anti-Lin28 antibodies, of day 8 hES cells treated with FGF2 isoforms (c). Commercially available FGF2 from Peprotech was used as a positive control. hES cell medium without exogenous FGF2 was used as a negative control (NO FGF2)

The morphology of hES colonies is maintained by all FGF2 treatments at day 8. There appears to be some differentiation in the periphery of the colonies in the control lacking FGF2 (Figure 3.7 a,b) at day 8 (white arrows). Analysis of stem cell gene expression by RT-qPCR and RT-PCR at day 18 after treatment, revealed that there is no major difference in gene expression levels between the different FGF2 treatments (Figure 3.8 a, b).

a. qRT-PCR



b. Traditional RT-PCR

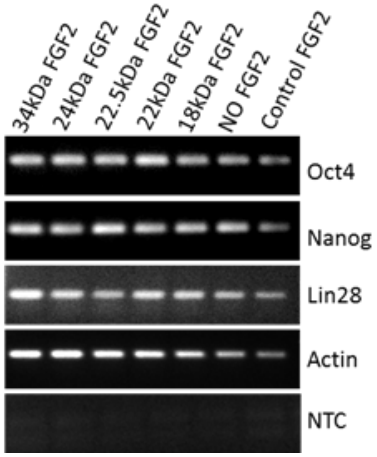
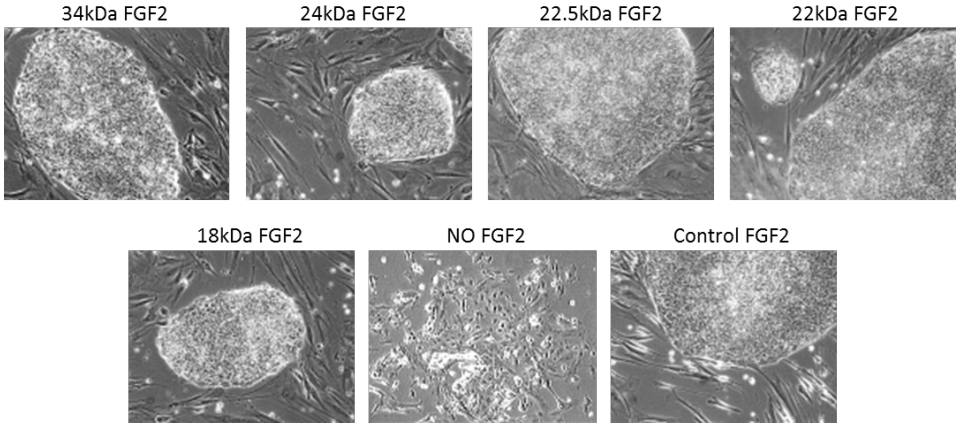


Figure 3.8 I **Day 18 RT-qPCR and traditional RT-PCR analysis**
RT-qPCR analysis of day 18 treated hES cells with FGF2 isoforms (a) and traditional RT-PCR analyzed on agarose gel (b). Commercially available FGF2 from Peprotech was used as a positive control. hES cell medium without suplemented FGF2 was used as a negative control.

Human ES cell colony morphology and analysis of protein expression by Western blot at day 18 after treatment shows loss of stem cell specific marker expression for the NO-FGF2 treated samples while expression in FGF2 isoform treated cells remains similar to the FGF2-control treated cells (3.8 a, b).

a.



b.

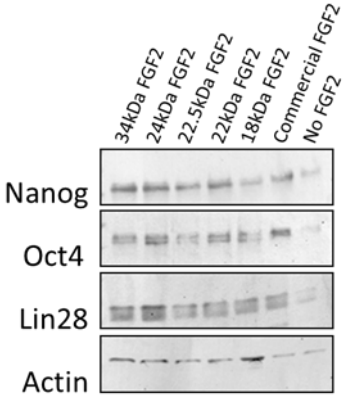


Figure 3.9 I **Day 18 Morphology and Western blot analysis** Morphology of day 18, FGF2 isoform treated hES cells (a); Western blot analysis with anti Oct-4, anti-Nanog and anti-Lin28 antibodies, of day 18 hES cells treated with FGF2 isoforms (b). Commercially available FGF2 from Peprotech was used as a positive control. hES cell medium without exogenous FGF2 was used as a negative control (NO FGF2)

Induction of stem cell protein expression and population doubling in human dermal fibroblasts

RT-PCR analysis with primers for stem cell genes was performed at day 8 and day 30 of treatment with FGF2 isoforms. The results demonstrated amplification of stem cell genes at both time points (Figure 3.11 a,b). Similar findings showing the expression of stem cell specific proteins in human dermal fibroblasts have been published from our lab for the 18kDa isoform (Page, Ambady et al. 2009). Western blot analysis at day 8 and day 30 of treatment with FGF2 isoforms showed induction of expression for stem cell specific proteins in cultures treated with FGF2 isoforms (Figure 3.11 c,d).

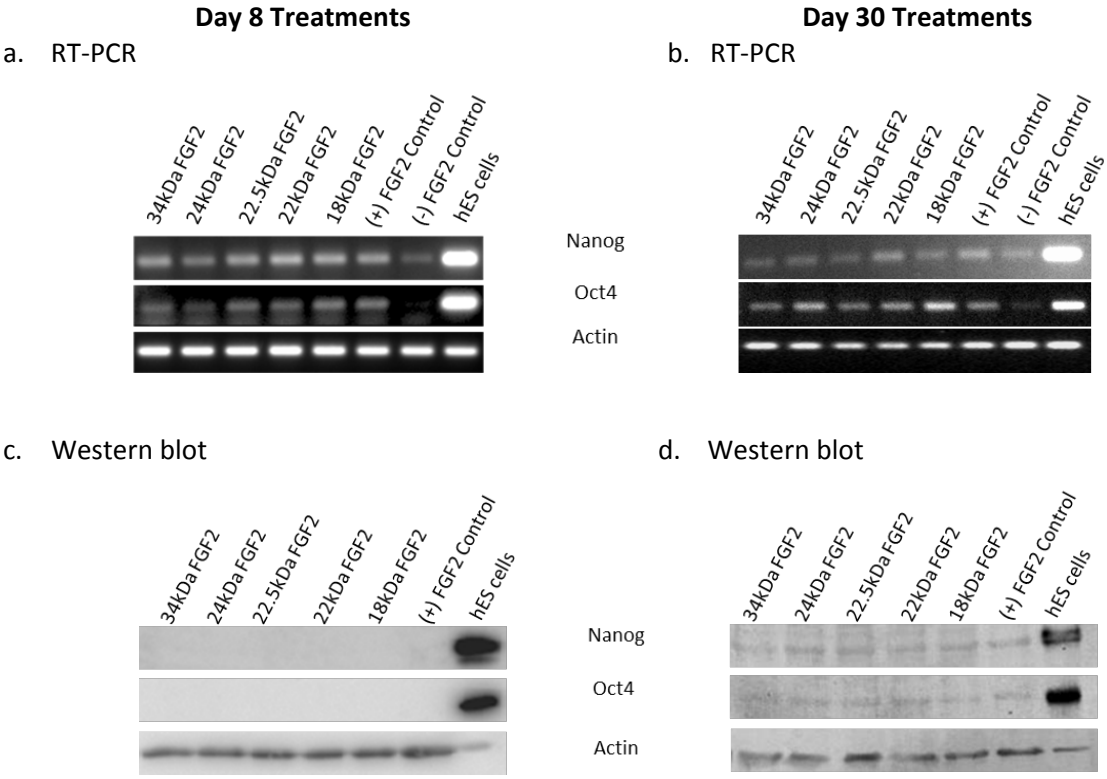
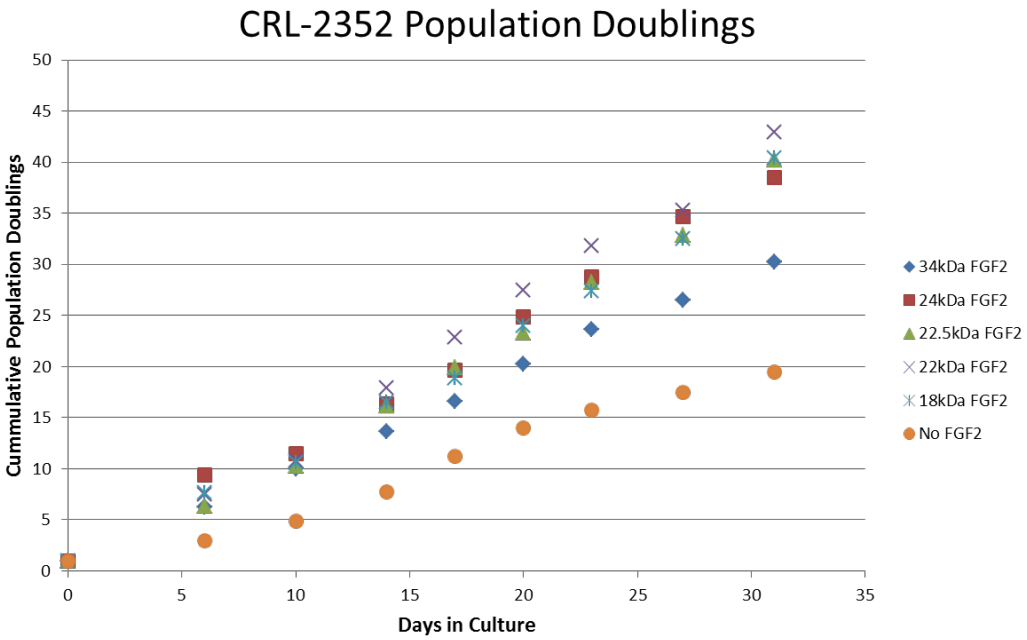


Figure 3.10 I **FGF2-isoform induction of stem cell specific protein expression in treated human dermal fibroblasts at day 8 and day 30** RT-PCR analysis of day 8 and day 30 human dermal fibroblasts treated with FGF2 isoforms (a,b). Western blot analysis of day 8 and day 30 human dermal fibroblasts treated with FGF2 isoforms (c,d). Indicated primary antibodies used for each target.

Adult human fibroblasts grown in DMEM/F12 (50/50), 10% Fetal Clone III (Hyclone), with 4ng/ml of various FGF2 isoforms at 37^{O}C and 5% O2, 5% CO2, 90% N2 were cultured continuously with a rigorously controlled passage schedule, and assayed for population doubling. Cells cultured with FGF2 isoforms underwent between 30-42 population doublings (PDs) during 32 days of culture compared to 19 PDs for cells cultured in 5% oxygen without FGF2 (Fig. 3.11). The increase in population doubling rates for the FGF2 isoforms supplementation was accompanied by change in morphology to smaller cells with a more spindle shape.



FGF2 isoform	Cumulative PD at Day 32
34 kDa FGF2	30.2
24 kDa FGF2	38.5
22.5 kDa FGF2	40.2
22 kDa FGF2	42.9
18 kDa FGF2	40.4
No FGF2	19.5

Figure 3.11 I **Cumulative population doubling over 30 days of treatment** CRL 2352 human dermal fibroblasts treated for 30 days with FGF2 isoforms or grown without FGF2.

Discussion

During this study we analyzed the biological activities of various recombinant FGF2 isoforms using adult human dermal fibroblasts and human ESCs. We first focused on establishing an FGF2 expression and purification process that would allow for production of highly pure, biologically active recombinant FGF2 isoforms. We showed that all five FGF2 isoforms ranging from 18kDa to 34kDa can be over-expressed as individual isoforms using the bac-to-bac® baculovirus expression system. Coomassie stained SDS-PAGE analysis indicates that individual isoforms can be isolated through a single IMAC chromatographic step with up to 85-90% purity. Our study also shows that all recombinant HMW FGF2 isoforms, exogenously supplemented in the culture medium, like the 18kDa isoforms, have the ability to

activate FGF receptor 1, as demonstrated by the phosphorylation state of the receptor. Moreover, all FGF2 isoforms phosphorylate FRS2α protein with similar efficiency in human dermal fibroblasts. HMW FGF2 isoforms can also equally stimulate ERK1/2 phosphorylation. Since the receptor binding activity of 18kDa FGF2 has been associated with its ability to maintain hES cells in an undifferentiated state (Eiselleova, Matulka et al. 2009, Ding, Ling et al. 2010), we investigated the ability of the HMW FGF2 isoforms to support hESC growth. Our results show that all FGF2 isoforms have the ability to maintain the expression of stem cell specific genes, stem cell specific proteins as and support the growth of human embryonic stem cells on inactivated mouse embryonic fibroblasts for at least 28 days. Moreover, the treatment of human dermal fibroblasts with individual FGF2 isoforms induces expression of stem cell specific proteins and extends the number of population doublings.

To our knowledge, this is the first study to analyze the ability of individual HMW FGF2 isoforms to activate signal transduction of the MAPK canonical pathway when HMW FGF2 isoforms are exogenously supplemented in the culture medium of human dermal fibroblasts in vitro. This study shows that all five FGF2 isoforms can be overexpressed as recombinant proteins and can be purified with high purity through a single chromatographic step. This is also the first study to analyze the ability of HMW FGF2 isoforms to support the growth of human embryonic stem cells in an undifferentiated state. This study may provide insight to why HMW FGF2 isoforms are secreted into the extracellular space. Findings from this work may provide insights supporting the hypothesis that HMW FGF2 isoform release into the extracellular space amplifies the 18kDa effect when higher levels of exogenous FGF2 are needed (Chlebova, Bryja et al. 2009).

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Abstract

Fibroblast Growth Factors are involved in many aspects of mammalian development, and determining binding specificity of FGF ligands to FGF receptor tyrosine kinases is very important in determining the role they play during development. Sequence differences between FGF receptors as a result of alternative splicing as well as sequence differences in FGF ligands are responsible for determining ligand-receptor specificity. FGF2 specifically, has been described as a factor important in wound response, nervous system development, angiogenesis and maintenance of embryonic stem cells in an undifferentiated state in vitro. Five FGF2 isoforms exist as a result of translation initiation at alternative start sites. Dissecting the interactions between the FGF2 isoforms and FGF receptor variants would help us to understand their function. To determine the relative mitogenic activity of specific ligand-receptor variants we created transgenic cell lines expressing recombinant receptor variants and assayed the mitogenic activity of the five FGF2 isoforms compared to commercial FGF1 as a control.

Introduction

Human dermal fibroblasts naturally express several isoforms of FGF2 as well as select FGF receptors (Hughes 1997, Sato, Segami et al. 2003, Sperger, Chen et al. 2003, Dvorak, Dvorakova et al. 2005, Eiselleova, Matulka et al. 2009). FGFR activation upon ligand binding results in the stimulation of various signal transduction cascades that have been implicated in multiple aspects of vertebrate and invertebrate embryonic development, tumor growth, angiogenesis, wound healing, and physiology (Powers, McLeskey et al. 2000, Ornitz and Itoh 2001, Ornitz and Marie 2002). Also, unregulated expression of FGFs has been shown to be implicated in cancer progression and development (Dailey, Ambrosetti et al. 2005, Ezzat and Asa 2005, Grose and Dickson 2005). The LMW (18kDa) isoform is the most well studied of the FGF2 ligands, and is reported to have intracrine, autocrine as well as paracrine activities (Arese, Chen et al. 1999, Strutz, Zeisberg et al. 2000). Binding of the FGF2 ligand to the

receptor triggers receptor dimerization, phosphorylation of its kinase domain, and activation of several intracellular kinase pathways which result in ERK1/2 phosphorylation. Investigation of the ability of each isoform to bind various receptors and activate downstream kinase pathways, and identification of FGF-FGFR binding specificities are critical to understanding the biological mechanisms involved in normal development and pathogenesis. Previous research has demonstrated that HMW FGF2 isoforms can be transported to the extracellular environment (Santiago, Ma et al. 2011), and the 24kDa FGF2 isoform administered exogenously to endothelial cells induces cell proliferation with a similar dose response as the 18kDa isoforms (Taverna, Ghersi et al. 2003). Here, we investigate the specificity of each FGF2 isoform for various FGF receptor tyrosine kinase variants.

Cell proliferation, migration, differentiation, metabolic homeostasis, wound repair and the development of multinuclear organisms are regulated by a variety of secreted polypeptides such as growth factors, hormones and cytokines. Several groups of these secreted polypeptides act by binding to and activating cell surface receptors with intrinsic tyrosine kinase activity. Fibroblast growth factors are part of a group of secreted polypeptides that use low affinity surface receptors (Ornitz, Yayon et al. 1992) as well as high affinity membrane spanning receptor tyrosine kinases that upon extracellular ligand binding activate intracellular domain functions (Ornitz, Herr et al. 1995). The low affinity FGF receptors include heparin and HSPGs. FGF receptor tyrosine kinases possess a glycosylated extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic tyrosine kinase domain (Hanks, Quinn et al. 1988) (Yarden and Ullrich 1988) responsible for initiating intracellular signaling (Figure 4.1a). Previous studies have shown that FGF binding to heparin-like low affinity receptors is required for binding to receptor tyrosine kinases and activation of downstream signaling pathways (Yayon, Klagsbrun et al. 1991). The binding of FGF to heparin has also been shown to protect FGF from degradation and increase the stability of the growth factor (Gospodarowicz and Cheng 1986). Five isoforms of FGF2, ranging from 18kDa to 34kDa exist as a result of translation initiation at alternative

start sites (Florkiewicz and Sommer 1989, Arnaud, Touriol et al. 1999). HMW FGF2 isoforms, 22kDa, 22.5kDa, 24kDa, and 34kDa, respectively, are co-linear extensions of the 18kDa isoform (Figure 4.1b) (Florkiewicz and Sommer 1989, Arnaud, Touriol et al. 1999).

a. b.

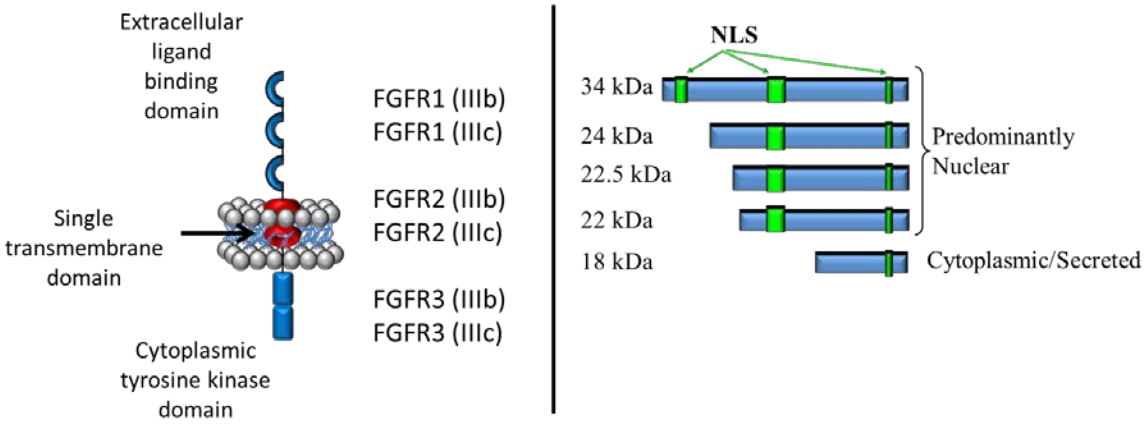

Figure 4.1 | **FGFR variants and FGF2 isoforms**
Genes coding for FGFR 1, FGFR2 and FGFR3 produce transmembrane proteins with an extracellular domain, single transmembrane domain and a cytoplasmic kinase domain. Two spliced variants for each receptor gene (IIIb and IIIc) give rise to transmembrane receptors with kinase activity (a). Five FGF2 isoforms exist. Four high molecular weight isoforms FGF2 isoforms are N-terminal extensions of the 18kDa isoform. HMW FGF2 isoforms contain additional nuclear localization sequence which target high molecular weight isoforms FGF2 isoforms to the nucleus of expressing cells. (b)

The HMW FGF2 isoforms are predominantly intracellular and localize to the nucleus, while the 18kDa isoform is mainly cytoplasmic and is secreted from cells by a process that is not fully understood (Figure 4.1b). The localization of FGF2 isoforms varies depending on cellular conditions and stimuli (Arese, Chen et al. 1999) and HMW isoforms have been described in some occasions to be present in the extracellular space, secreted through a process known as vesicle shedding (Taverna, Ghersi et al. 2003). Four distinct genes have been identified to encode FGF receptors (FGFR1, FGFR2, FGFR3 and FGFR4) (Lee, Johnson et al. 1989, Ruta, Burgess et al. 1989, Dionne, Crumley et al. 1990, Hattori, Odagiri et al. 1990, Reid, Wilks et al. 1990, Safran, Avivi et al. 1990, Partanen, Makela et al. 1991, Yayon, Klagsbrun et al. 1991) and mRNA splicing produces several receptor variants for each receptor, which exhibit

selective binding properties for different FGFs (Champion-Arnaud, Ronsin et al. 1991, Miki, Bottaro et al. 1992, Werner, Duan et al. 1992). The variety of FGF ligands and FGFR variants provides a high level of diversity in ligand specificity and biological function. Previous research has demonstrated that HMW FGF2 isoforms can be transported to the extracellular environment (Presta, Gualandris et al. 1993) and the 24kDa FGF2 isoform administered exogenously to endothelial cells induces cell proliferation with a similar dose response as the 18kDa isoform (Taverna, Ghersi et al. 2003). The identification of FGF2-FGFR binding specificities is critical to understanding the biological mechanisms involved in normal development and pathogenesis.

Materials and Methods

Preparation of endotoxin free MIRB-FGFR plasmid for transfection

FGF receptor variants in MIRB vector were transformed into chemically competent *E.coli.* Cells were harvested by centrifugation at 3000 x g, 24 hours after growing at 37^{o}C with shaking at 250 rpm. Cells were resuspended (1 : 10 :: weight : volume) in buffer P1 (50mM Tris-HCl pH 8.0, 10mM EDTA, 100µg/ml RNase A). Cells were lysed in an equal volume of lysis buffer (200mM NaOH, 1% SDS) for 30 minutes on ice. The lysate mixture was neutralized in neutralization buffer (3.0M potassium acetate pH 5.5) and centrifuged at 9,000 x g for 60 minutes. Supernatant was filtered to remove any particles and then incubated on ice for 15 minutes after addition of 1/10 volume of endotoxin removal buffer (1% Triton X-114). Ion exchange resin from Qiagen was hydrated and equilibrated in column loading buffer (750mM NaCl, 50mM MOPS, 15% isopropanol, 0.15% triton X-100, pH 7.0). The sample was loaded by gravity flow, and resin was washed with 10 column-volumes of wash buffer (1.0mM NaCl, 50mM MOPS, 15% Isopropanol, pH 7.0). Plasmid was eluted in 20 mL elution buffer (1.25mM NaCl, 50mM Tris-HCl, 15% isopropanol pH 8.5). Plasmid was precipitated by centrifugation at 9,000 x g with 0.7 volumes of isopropanol. The sample was washed twice in 70% endotoxin-free ethanol, air dried and resuspended in

endotoxin free water. Prior to transfection, 50 µg of plasmid were linearized by digesting with 50 units of ClaI (New England Biolabs) restriction enzyme for 1 hour at 37^{o}C. The final plasmid concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fischer).

Overexpression of FGF receptor variants in BaF3 cells

BaF3, an immortalized murine bone marrow-derived pro-B-cell line whose growth and proliferation is dependent on the presence of IL-3 ligand was used to test FGF2 ligand activity. BaF3 cells do not express endogenous FGF receptor tyrosine kinases and do not normally demonstrate a biological effect in the presence of extracellular FGF ligands. Transgenic BaF3 cells overexpressing FGF receptors can bind extracellular FGF ligands, can activate intracellular pathways and can proliferate in the absence of IL-3 if the supplemented FGF ligand demonstrates activity. Three receptor variants, FGFR1 IIIb, FGFR2 IIIb and FGFR3 IIIb, respectively, were transfected into wild type BaF3 cells individually (Figure 4.2).

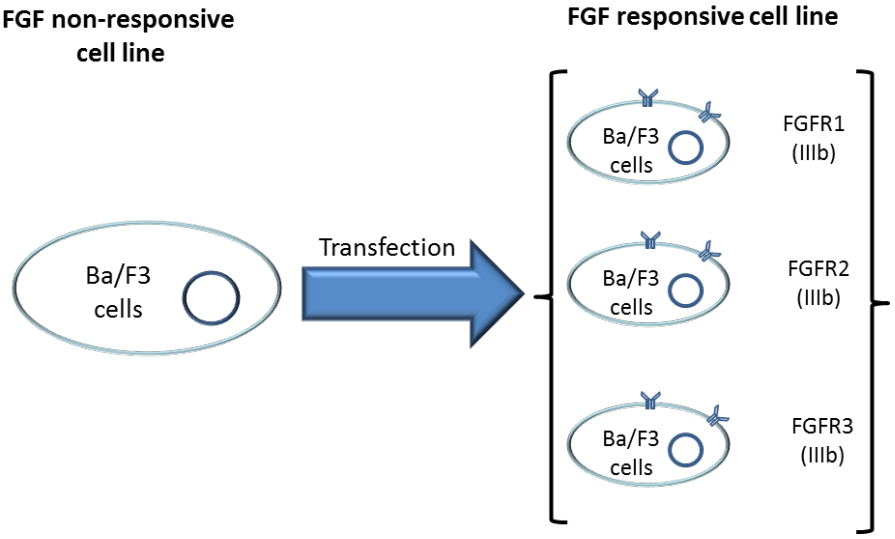

Figure 4.2 | **Schematic representation of expression of FGFR variants in BaF3 cell line** Each receptor variant is individually transfected in BaF3 cells and a clonal population of stably transfected BaF3 cells is created for each receptor variant.

BaF3 cells were grown in suspension following published protocols (Ornitz, Xu et al. 1996) in RPMI 1640 media (Corning), supplemented with 10% FBS (HyClone), L-glutamine (HyClone), 50nM β-

Mercaptoethanol (Life Technologies), 10ng/ml IL-3 (Thermo Scientific) and 1X penicillin/streptomycin (Sigma Aldrich). Previously published electroporation protocols (Werner, Duan et al. 1992, Ornitz, Xu et al. 1996) were used to electroporate 20µg of ClaI-linearized MIRB-FGFR plasmid into 10^6 cells (Dell and Williams 1992, Ornitz and Leder 1992, Ornitz, Yayon et al. 1992, Ornitz, Xu et al. 1996) using a Gene Pulser Xcell™ Electroporation System (Bio Rad). Cells were grown in media without selection pressure for 16-18 hours, then selected in BaF3 complete growth media containing 600 µg/ml G418 (Life Technologies). Clonal cell lines for each receptor variant were established by limiting dilutions in a 96 well plate. The clonal cell lines used for the following experiments were evaluated for mitogenic responsiveness to FGF1 (Peprotech).

Total RNA extraction and RT-PCR

Total RNA was isolated from stably transfected and control BaF3 cells using TRIzol reagent (Life Technologies) following the manufacturer's protocol. One microgram of RNA was subjected to DNase digestion, followed by reverse transcription using qScript™ cDNA Synthesis Kit (Quanta Biosciences), according to manufacturer's protocol. PCR was performed using GoTaq® green master mix (Promega) with 1 µl of DNA template, 0.5 µl forward primer (10 µM stock), 0.5 µl reverse primer (10 µM stock) in a 25 µl reaction. PCR cycling was performed as follows: initial denaturation at 95°C for 7 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds; annealing at primer-specific annealing temperature for 1 minute; and extension at 72°C for 20 seconds. Final extension was done at 72°C for 7 minutes, and the samples were held at 4°C until use. Amplification products were resolved on 2% agarose gels containing 0.5 µg/ml ethidium bromide in 1X TAE buffer and imaged using a Bio Rad Gel Doc™ XR+ system (Bio Rad). Primer sequences used in this study are presented in Table 4.1.

Table 4.1 | **Primer sets used for amplification of FGF receptor variants**

Gene name	Forward Primer	Reverse Primer	Amplicon size (bp)
FGFR1 IIIb	CTTGACGTCGTGGAACGATCT	CACGCAGACTGGTTAGCTTCAC	320
FGFR2 IIIb	CCCATCCTCCAAGCTGGACTG	CAGAGCCAGCACTTCTGCATTG	216
FGFR3 IIIb	CAAGTTTGGCAGCATCCGGCAGAC	TCTCAGCCACGCCTATGAAATTGGTG	336

SDS-PAGE and Immunoblotting

A 0.2g cell pellet from each FGFR stably transfected culture and an untransfected control was lysed in 200µl cold lysis buffer supplemented with protease inhibitor cocktail. Cells were lysed on ice using a Missonix XL-2000 ultrasonic cell disruptor by 3 x 10 pulses at power 2. Total cell lysate was sampled in 5X Laemmli gel loading buffer. A clarified soluble fraction was generated by centrifugation at 14,000 x g for 30 minutes at 4^{o}C. Samples were mixed with 5X gel loading buffer then boiled for 5 minutes, and proteins separated by gel electrophoresis on 4-20% SDS-PAGE gel using a Bio-Rad Mini Protean system. For SDS-PAGE analysis the gel was stained with coomassie brilliant blue stain. For Western blot analysis the proteins were transferred to PVDF membrane (Millipore) using a semi dry transfer apparatus (GE Healthcare). The membrane was blocked in TBST buffer (25mM Tris-HCl pH7.5, 130mM NaCl, 0.1% Tween-20) with 5% fat-free dry milk for 30 minutes. The membrane was incubated with anti-FGFR specific primary antibodies overnight at 4^{o}C. After washing the membrane 3 x 10 minutes, the membrane was incubated in alkaline phosphatase conjugated secondary antibodies for 2 hours at room temperature. The bands were visualized using Western blue reagent (Promega). Images were acquired with a Canon LiDE 200 scanner.

Mitogenic Assay

Mitogenic activity was defined as the ability of FGF ligands to cause a cell to transition from G1 to S phase of the cell cycle, and was determined by BrdU incorporation followed by staining with anti-BrdU antibody. Stably transfected BaF3 cells and untransfected controls were grown in suspension in

RPMI 1640 media (Corning), supplemented with 10% FBS (Hyclone), L-glutamine (HyClone), 50nM β-Mercaptoethanol (Life Technologies), 10ng/ml IL-3 (Thermo Scientific) and 1X penicillin/streptomycin (Sigma Aldrich). Labeling with bromodeoxyuridine (BrdU) after treatment with individual FGF2 isoforms was used to determine the percentage of cells progressing to S-phase (mitogenic assay) for each FGF2-FGFR variant interaction (Figure 4.3). Wild type BaF3 cells in the absence of IL-3, and FGF receptor-expressing BaF3 transgenic cells that do not respond to FGF2 ligands, lack the ability to progress through the cell cycle.

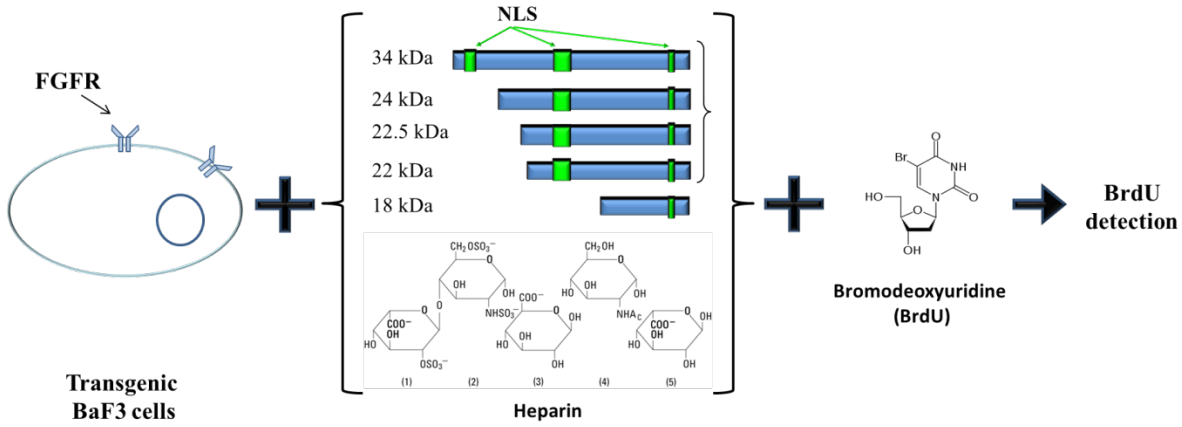

Figure 4.3 | **Schematic representation of cell proliferation assay (mitogenic activity)**
Stably transfected BaF3 cells expressing individual FGFR variants were incubated with individual FGF2 isoforms and 2 µg/ml heparin. BrdU reagent was added to the culture media 36-48 hours after treatment and cells were fixed, stained with anti BrdU antibody and analyzed by flow cytometry 6 hours after BrdU addition.

For the mitogenic assay, wild type BaF3 cells and stably transfected BaF3 cells were washed twice in RPMI media devoid of IL-3. Wild type cells and stably transfected BaF3 cells were plated in 6-well plates at 2 x 10^5 cells per well with the indicated growth factor concentrations (Table 4.2) and 2 µg/ml heparin.

Table 4.2 | **Concentration of FGF2 isoforms used for mitogenic activity assay**

	34 kDa	24 kDa	22.5 kDa	22 kDa	18 kDa	FGF1 (+)
	Concentration of FGF2 isoforms and positive control					
FGFR 1b	300 (pM)		1250 (pM)		5560 (pM)	
FGFR 2b	300 (pM)		1250 (pM)		5560 (pM)	
FGFR 3b	300 (pM)		1250 (pM)		5560 (pM)	

This concentration of heparin has been shown to be optimal for FGF1 binding to the receptors (Ornitz and Leder 1992, Ornitz, Herr et al. 1995, Santos-Ocampo, Colvin et al. 1996), and does not inhibit FGF2 activity (Ornitz, Xu et al. 1996). All cultures were incubated at 37°C, 5% O_2, 5% CO_2 and high humidity for 36-48 hours. BrdU labeling reagent (Zymed laboratories) was added to each treatment following manufacturer's suggestions, and cells were incubated at 37°C for 6 hours. Cells were centrifuged at 1,000 x g for 5 minutes, washed twice in Dulbecco's phosphate buffered saline (DPBS) then fixed in ice cold methanol for 20 minutes at 4°C. After rinsing with DPBS, cells were permeabilized with 1.5N HCl at 37°C for 10 minutes, and washed three times with PBS-Tween, 5 minutes each. Cells were incubated for 30 minutes at room temperature in primary anti-BrdU antibody (Developmental Studies Hybridoma Bank, Cat# G3G4) diluted 1:200 in PBS-Tween and. Cells were washed three times 5 minutes each in PBS-Tween, then incubated for 30 minutes in secondary Alexafluor488 goat anti-mouse antibody (Molecular Probes), diluted 1:500 in PBS-Tween. Cells were washed three times 5 minutes each in PBS, resuspended in 250µl PBS then subjected to flow cytometry analysis using a BD Accuri™ C6 flow cytometer. Transgenic BaF3 cells expressing FGFR variants treated with FGF1 were used as a positive control, and the same cells without any growth factor supplements were used as negative controls. Twenty thousand events were collected for each reaction.

Statistical analysis for Mitogenic Activity

The relative mitogenic activity for all FGF2 isoforms on individual FGFR variants was assessed through comparison with FGF1 activity. FGF1 is considered a positive control for FGF mitogenic assays, as previous studies have shown that FGF1 mitogenic activity to be the same regardless of the receptor variants present (Ornitz, Xu et al. 1996). Relative mitogenic activity was averaged at three different concentrations (312pM, 1250pM and 5500pM) to reduce sampling error (Table 4.1). The highest concentration chosen ensures the highest mitogenic activity level while cells are still in the near-log phase. The ranges of concentrations chosen are such that the mitogenic activity is as high as possible, yet the cells are still in near-log phase.

Results

Overexpression of FGF receptor variants in BaF3 cells

BaF3 cells electroporated with plasmid DNAs containing FGFR variants were cultured in growth media under selection pressure to generate stably transfected clonal populations. RT-PCR analysis with primers specific to FGF receptors (Table 4.1) shows the presence of FGFR mRNAs of the expected length in all stably transfected cultures (Figure 4.4a). A signal for FGF receptor variants is not present in untransfected BaF3 cells, which do not express any of the receptors endogenously. Western blot analysis of cell pellets from FGFR stably transfected cultures, and untransfected controls show protein expression at the expected molecular weights (70-90kDa) only for FGFR transfected samples and not in the untransfected control (Figure 4.4b). The presence of multible bands in transfected BaF3 cells has been described to be a result of the receptor being in different glycosylation states (Duchesne, Tissot et al. 2006). Western blot analysis is shown only for FGFR1 IIIb and FGFR3 IIIb. No working antibody was available for FGFR2 IIIb.

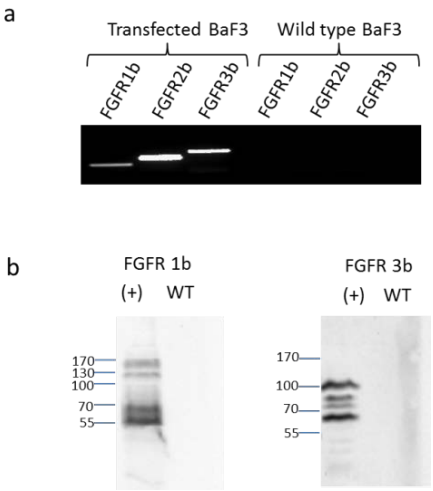

Figure 4.4 | **RT-PCR and Western blot analysis for overexpression of FGFR variants in BaF3 cells** RT-PCR analysis with primers specific to FGFR1, FGFR2 and FGFR3 respectively. Transfected and wild type BaF3 cells were analyzed for each receptor variant (a). Western blot analysis with antibodies specific to FGFR1 and FGFR3. Secondary alkaline phosphatase conjugated antibody was used for detection with Western blue reagent (b).

Mitogenic activity assay

All human FGF2 isoforms were virally expressed in an insect cell line and purified over Ni-NTA resin utilizing the 6xHis tag fused to each FGF2 isoform as described in chapter 3. To directly compare the activity of each individual FGF2 isoform with single FGFR variants, different concentrations of FGF2 ligands were assayed on FGFR-expressing BaF3 cells and monitoring the entry of cells into S-phase by BrdU incorporation after 36-48 hours treatment (Figure 4.3) and running subsequent flow cytometry analysis (Appendix C). Concentrations of FGF2 ranging between 300pM to 5500pM were used for these assays (Table 4.1). Flow cytometry analysis for BrdU incorporation demonstrates that all three FGFR expressing cell lines respond to positive control FGF1. This result is consistent with previous publications (Dionne, Crumley et al. 1990, Dell and Williams 1992, Werner, Duan et al. 1992, Ornitz, Herr et al. 1995), and confirms that FGF1 is a universal FGFR ligand and can validly be used as a positive control. Flow cytometry analysis for BrdU incorporation upon treatment of transgenic BaF3 cells with increasing concentrations of FGF2 ligands shows increased number of cells entering S-phase for all FGF receptor variants tested (Figure 4.5, 4.6, 4.7, Appendix C). However, the interaction of FGF2 ligands with the FGFR1 IIIb receptor variant displays the highest levels of relative mitogenic activity (Figure 4.8, Table 4.3).

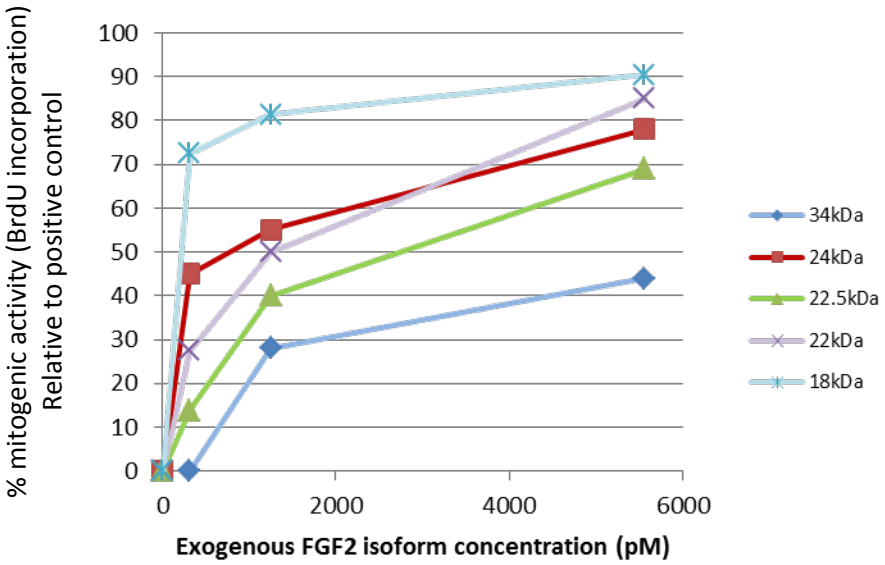

Figure 4.5 | **Graphic representation of relative mitogenic activity for FGFR1 IIIb** Relative mitogenic activity for FGF2 isoforms interacting with receptor variant FGFR1 IIIb at increasing ligand concentrations

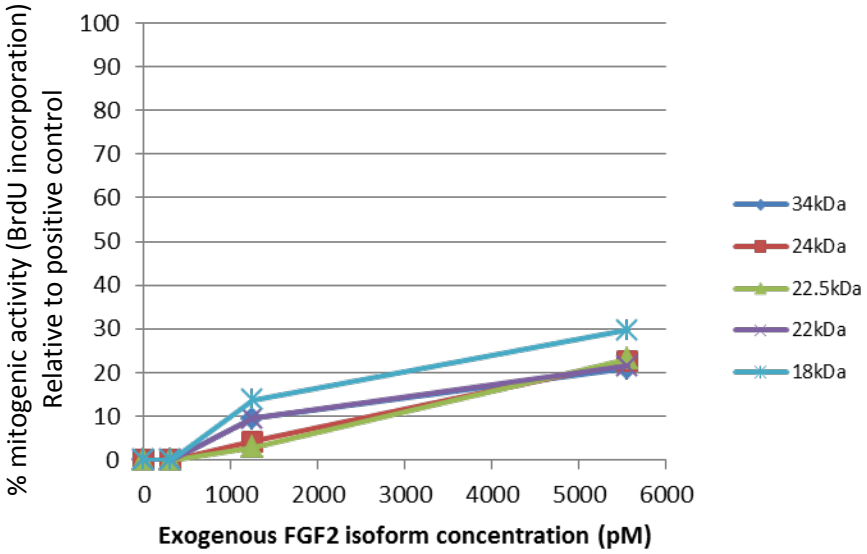

Figure 4.6 | **Graphic representation of relative mitogenic activity for FGFR2 IIIb** Relative mitogenic activity for FGF2 isoforms interacting with receptor variant FGFR2 IIIb at increasing ligand concentrations

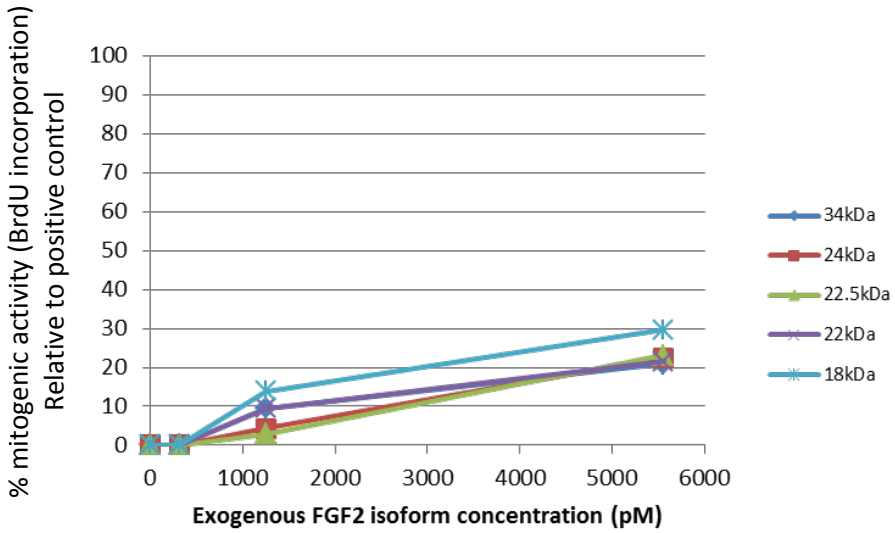

Figure 4.7 | **Graphic representation of relative mitogenic activity for FGFR3 IIIb**
Relative mitogenic activity for FGF2 isoforms interacting with receptor variant FGFR3 IIIb at increasing ligand concentrations

For all three FGF receptors, the low molecular weight FGF2 isoform displays the highest relative mitogenic activity when compared to the other isoforms. The relative mitogenic activity for the 18kDa FGF2 ligand is in agreement with previous publications (Ornitz, Xu et al. 1996). The level of relative mitogenic activity differs for different FGFR variants (Table 4.3), suggesting that the N-terminal extensions may interfere with mitogenic activity of FGF2 isoforms. Our results show that there is a statistical difference in mitogenic activity displayed from all FGF2 isoforms interacting with FGFR1 IIIb and FGFR2 IIIb (Figure 4.8). No statistical difference in relative mitogenic activity is observed between HMW FGF2 isoforms reacting with FGFR3 IIIb, except for the 18kDa isoform. All FGF2 isoforms demonstrate low relative mitogenic activity when reacting with FGFR IIIb. A summary of the relative mitogenic activity for all FGF2 isoforms tested is presented in Table 4.3.

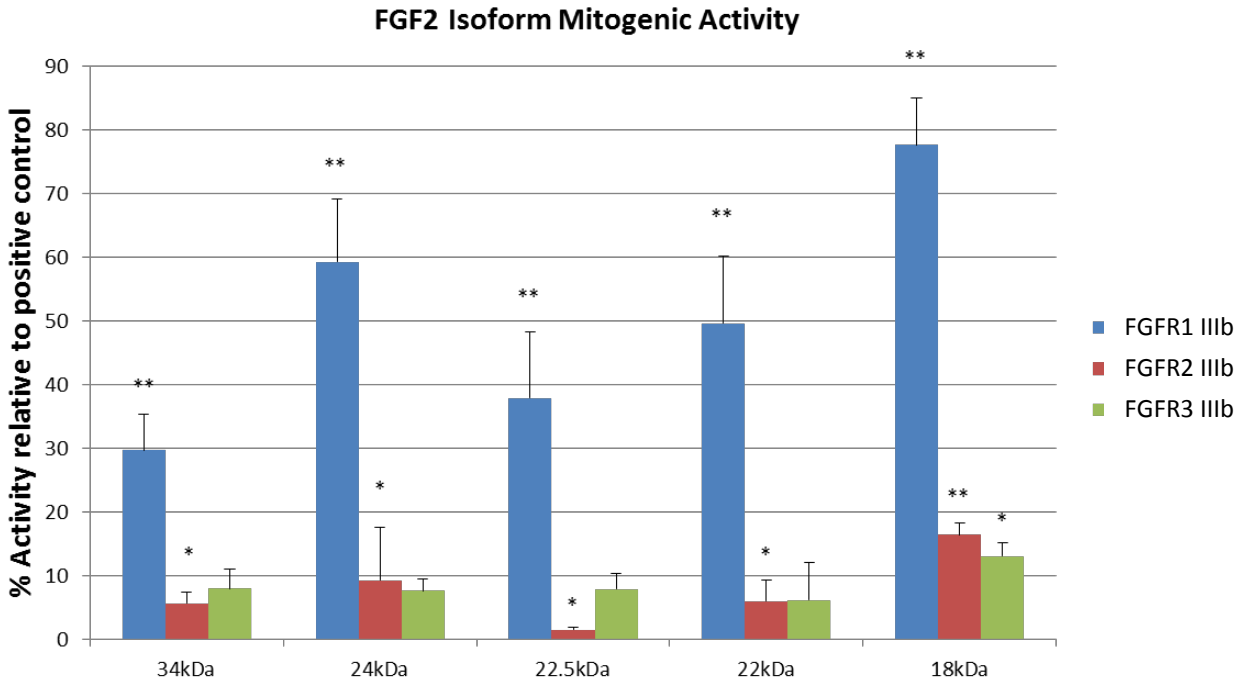

Figure 4.8 | **Graphic representation of % mitogenic activity relative to positive control**
Mitogenic activity is compared between isoform treatments for the same FGF receptor variants, respectively, using one way analysis of variance (ANOVA), alpha=0.05. Error bars indicate Standard deviation (SD). $P < 0.05$ *; $P < 0.01$ **

Table 4.3 | **Summary of relative mitogenic activity for all FGF2 isoforms and three receptor variants**

FGF receptor	FGF ligand											
	FGF1 ± S.D.		34kDa FGF2 ± S.D.		24kDa FGF2 ± S.D.		22.5kDa FGF2 ± S.D.		22kDa FGF2 ± S.D.		18kDa FGF2 ± S.D.	
1 IIIb	100.0	4.5	29.7	5.6	59.3	9.8	37.9	10.3	49.6	10.6	77.6	7.3
2 IIIb	100.0	1.9	5.7	1.8	9.3	3.2	1.5	0.4	6.0	3.3	16.4	1.8
3 IIIb	100.0	2.1	7.9	3.1	7.6	1.8	7.8	2.6	6.1	5.9	13.0	2.0

Statistical analysis comparing the relative mitogenic activity between different FGF2 isoforms for the same receptor variants shows a significant difference for receptors FGFR1 IIIb and FGFR2 IIIb but not for FGFR3 IIIb. The FGF2 mitogenic activity for all FGF2 isoforms binding to FGFR3 IIIb is very low. This data

agrees with previous publications where low levels of mitogenic activity are reported for 18kDa FGF2 isoform binding to FGFR3 IIIb (Ornitz, Xu et al. 1996).

Discussion

The FGF family has 22 members, many of them expressing several isoforms, and four FGF receptors, three of them expressing several splice variants. The large number of FGF ligands and their high level of promiscuity for specific FGF receptor variants provide a very high level of ligand-receptor diversity. FGF ligands and their receptors are expressed in a developmental stage-dependent manner as well as in a tissue-specific pattern. Missexpression of FGF ligands and FGFR variants has been involved in several cellular processes and diseases [reviewed in (Eswarakumar, Lax et al. 2005)]. The role that FGF ligands and their receptors play in development and disease requires a better understanding of the interaction and specificity of each FGF ligand with specific FGFR variants. The activity of HMW FGF2 isoforms as intracellular factors has been studied by many groups and a large body of work has been published describing their complex role in cellular processes. However, here we describe a different role for HMW FGF2 isoforms as extracellular factors. BrdU flow cytometry analysis used to quantitate the mitogenic activity of all FGF2 isoforms demonstrated relatively high binding specificity and activity for all FGF2 isoforms when paired with FGF receptor 1 IIIb, which suggests that all FGF2 isoforms preferentially bind FGF receptor 1 IIIb with higher specificity than FGF receptor 2 IIIb or FGF receptor 3 IIIb. Statistical analysis comparing the activity of the FGF2 isoforms for specific receptor variants revealed a significant difference of 10% or higher in mitogenic activity when FGF2 isoforms are paired with FGF receptor 1 IIIb, with the 18kDa FGF2 isoform showing the highest activity. The analysis also showed that FGF2 isoforms demonstrate slightly higher activity when interacting with FGF receptor 2 IIIb than FGF receptor 3 IIIb. This information could be important when studying effect of FGF ligands on different cell types or switches in FGFR expression patterns induced by presence of different FGF ligands. Understanding

receptor binding specificities for various FGF family members and the FGF receptor expression patterns, allows a prediction of the level of mitogenic activity that FGF ligands might have on specific cell types when either one or multiple receptor variants are expressed. The data presented in this study shows the ability of HMW FGF2 isoforms to act as extracellular ligands for select FGF receptor tyrosine kinases and to activate the mitogenic pathways that support cell proliferation in vitro. This work shows that HMW FGF2 isoforms can react with FGF receptor tyrosine kinases with nearly similar activity as the 18kDa FGF2 isoform, although the N-terminal extensions present in HMW FGF2 isoforms may slightly lessen their mitogenic activity. The 18kDa FGF2 isoform displays the highest level of mitogenic activity when compared with the HMW FGF2 isoforms. However, the length of N-terminal extensions does not seem to correlate with a decrease in mitogenic activity for HMW FGF2 isoforms. This study suggests that the extracellular HMW FGF2 isoforms may have a role that is distinct and independent of their intracellular role, and that their signaling capabilities could be important even in the absence of the LMW FGF2.

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Abstract

FGF2 is a member of the FGF family known as heparin binding factors and was first purified as a heparin-binding molecule from bovine pituitary (Gospodarowicz et al., 1984). FGFs demonstrate their biological activity through the activation of surface FGF receptors (Ornitz et al., 1996; Zhang et al., 2006), and possess broad mitogenic and angiogenic activity. Five isoforms of FGF2, ranging from 18kDa to 34kDa exist as a result of translation initiation at alternative start sites (Arnaud et al., 1999; Florkiewicz and Sommer 1989). HMW FGF2 isoforms, 22kDa, 22.5kDa 24kDa and 34kDa respectively, are co-linear extensions of the 18kDa isoform (Arnaud et al., 1999; Florkiewicz and Sommer 1989). The HMW isoforms are predominantly intracellular and localize to the nucleus, while the 18kDa isoform is mainly cytoplasmic and is secreted from cells by a process that is not fully understood. Localization of the FGF2 isoforms varies depending on the cellular conditions and stimuli (Arese et al., 1999) and sometimes the low molecular weight isoform is translocated to the nucleus. A few nuclear targets have been described for select HMW FGF2 isoforms, and the quest for identifying new targets is ongoing. Here we identify E3 ubiquitin ligase HECW1 as a new gene target for FGF2 isoforms.

Introduction

Several growth factors such as ciliary neurotrophic factor, platelet-derived growth factor (PDGF), FGF1 and FGF2 as well as their respective receptors can localize into the nuclei of expressing cells (Claus et al., 2003; Jans and Hassan 1998; Pederson 1998). When present in the nucleus, these factors may assume a function that is independent of their extracellular function. Translational initiation at alternative CUG start sites produces four high molecular weight isoforms of FGF2 with molecular weights of 22, 22.5, 24 and 34kDa which are linear extensions of the 18kDa isoform translated from the AUG codon (Arnaud et al., 1999; Florkiewicz and Sommer 1989; Prats et al., 1989). The 18kDa isoform

can be secreted from the expressing cells, and demonstrates its activity by binding and activation of surface FGF receptors (Baird 1994; Powers et al., 2000). Nuclear translocation of the low molecular weight FGF2 has also been observed (Baldin et al., 1990; Choi et al., 2000) but the role of the 18kDa FGF2 in the nucleus is not fully understood. A shared bipartite NLS is located on the carboxy-terminal end of all FGF2 isoforms (Foletti et al., 2003). Additional NLS sites found on HMW isoforms are located on the amino-terminal extensions not present in the 18kDa isoform (Rifkin et al., 1994). On the other hand, HMW FGF2 isoforms predominantly translocate in the nucleus (Arese et al., 1999) in a NLS dependent manner (Claus et al., 2003). FGF2 isoforms have been shown to be involved in embryonic development, wound healing, cell growth, cell migration, apoptosis, cell differentiation, oncogenesis etc., and many effects of FGF2 have been well characterized; however only a limited number of nuclear binding partners and gene targets have been identified, such as anti-apoptotic protein Api1 (Krejci et al., 2007), ribosomal protein L6/TAXREB107 (Klein et al., 1996; Renko et al., 1990), splicing factor SF3a66 (Gringel et al., 2004), survival of motor neurons protein (Claus et al., 2003) promoter region of bone sialoprotein (Boudreaux and Towler 1996; Shimizu-Sasaki et al., 2003). Identifying and understanding the role of growth factor nuclear localization and the nuclear interactions that control the cellular mechanisms they are involved in will increase our understanding of their mode of action and allow us to better control cell fate. Here we describe our efforts to identify nuclear DNA binding targets for individual FGF2 isoforms (Figure 5.1) and identify a novel nuclear target.

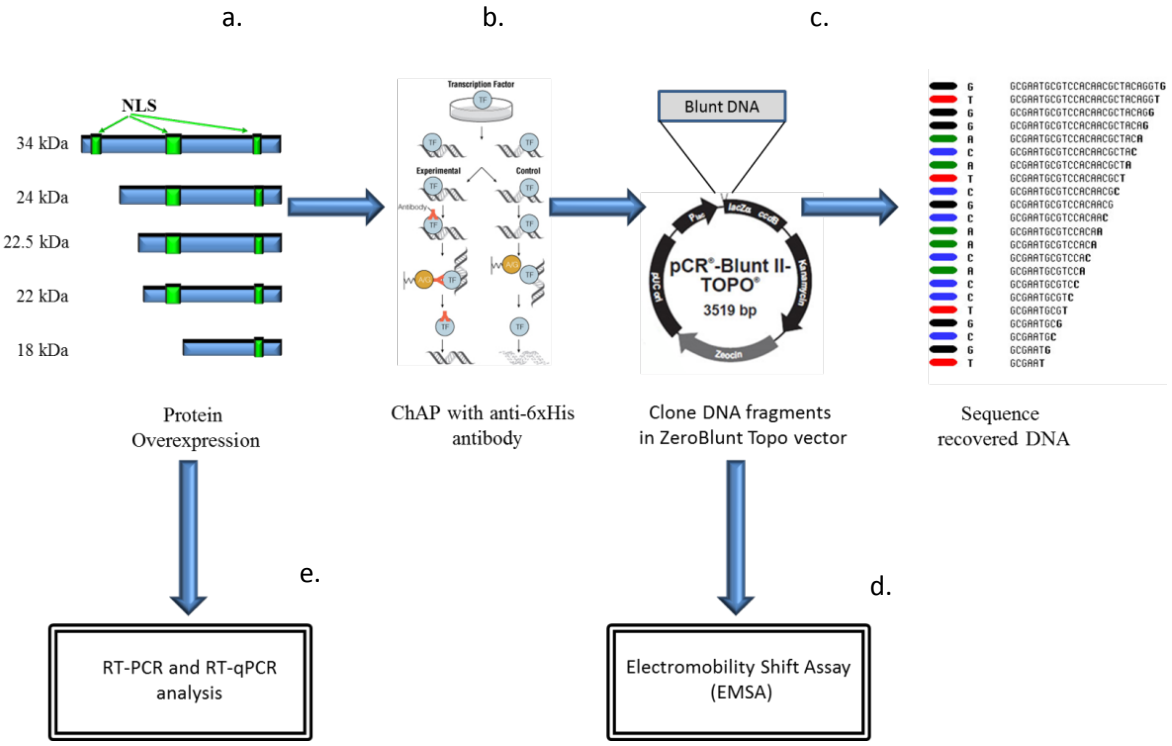

Figure 5.1 | **Experimental outline for identification of DNA binding targets for FGF2 isoforms** Protein over-expression in HeLa (a) followed by chromatin affinity purification to isolate FGF2 protein-DNA complexes (b). Isolated DNA is cloned in PCR ZeroBlunt Topo II vector to help with sequencing and identification of unknown DNA fragments (c). Electromobility shift assay to test *in vitro* for binding of FGF2 isoforms to DNA sequences (d). RT-PCR and RT-qPCR analysis to validate the effect of FGF2 isoforms on target gene expression (e).

All five FGF2 isoforms were individually sub-cloned into the pLVX-Puro mammalian expression vector, and their expression in HeLa cells was verified by Western blot analysis. Chromatin immunoprecipitation analysis followed by DNA sequencing identified the promoter region of E3 ubiquitin ligase (HECW1) gene as a potential target for one of the high molecular weight FGF2 isoforms. Binding of this isoform to the identified sequence was confirmed by PCR analysis of the target gene pre and post transfection was used to identify any effects of binding on the expression levels of HECW1.

Materials and Methods

Sub cloning of FGF2 isoforms in pLVX-Puro mammalian expression vector

All five FGF2 isoforms were individually synthesized and subcloned in the pBluescript vector (Epoch labs). Alternative start sites were mutated to ensure the expression of single isoforms from each clone and each FGF2 isoform was designed as a 6xHis fusion protein with a TEV cleavage site (Described in chapter 3). Gene inserts were isolated from the pBluescript vector by first digesting with HindIII restriction enzyme (New England Biolabs), endfilling with DNA Polymerase I large (Klenow) Fragment (New England Biolabs) then digesting with EcoRI enzyme (New England Biolabs). The gene inserts were isolated through gel extraction on 1% agarose gels (Figure 5.2 a) using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The pLVX-Puro vector (Clonetech) was digested with SmaI enzyme (New England Biolabs) and endfilled using DNA Polymerase I large (Klenow) Fragment (New England Biolabs). The vector was then digested with EcoRI enzyme (New England Biolabs) in order to ensure directional ligation of the gene of interest. The vector DNA was dephosphorylated using Calf Intestinal Phosphatase (CIP) to reduce self-ligation. Vector DNA was gel purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). T4 DNA ligase (New England Biolabs) was used to ligate the FGF2 genes into pLVX-Puro vectors. Endotoxin-free plasmid for each FGF2 construct was generated, and directional insertion of the DNA fragment was confirmed by restriction digestion.

FGF2 isoform Over-expression

HeLa cells were grown in 10 cm tissue culture dishes at 70% confluency prior to transfection with FGF2 isoforms. Plasmid DNAs containing inserts for individual FGF2 isoforms were transfected to HeLa cells using Lipofectamine® 2000 (Life technologies). Cells were incubated with lipofectamine/plasmid DNA in 50/50 DMEM : 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 : 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. Cells were harvested 36 hours post transfection, and RNA was isolated for RT-PCR and qRT-PCR analysis.

RT-PCR and RT-qPCR assay

RNA from transfected and untransfected cells was extracted using TRIzol reagent (Life Technologies) according to the manufacturer's protocol, and quantified by spectrophotometry using a Nanodrop 2000 spectrometer (Thermo Scientific). One microgram of RNA was subjected to DNase digestion, followed by reverse transcription using qScript™ cDNA Synthesis Kit (Quanta Biosciences), according to the manufacturer's protocol. RT-PCR was performed using GoTaq® green master mix (Promega) with 1 µl of cDNA template, 0.5 µl forward primer (10 µM stock), 0.5 µl reverse primer (10 µM stock) in a 25 µl reaction. PCR cycling was done as follows using a Bio-Rad thermo cycler: initial denaturation at 95°C for 7 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at primer-specific annealing temperature for 1 minute, and extension at 72°C for 20 seconds. Final extension was done at 72°C for 7 minutes, and the samples were held at 4°C until use. Amplification products were resolved on 2% agarose gels containing 0.5 µg/ml ethidium bromide in 1X TAE buffer and imaged using a Bio Rad Gel Doc™ XR+ system (Bio Rad). Twenty ng/well of cDNA was used as a template in RT-qPCR reactions with intron-spanning primers specific for the genes of interest (Table 5.1).

Table 5.1 | **Primer set used for identification of HECW1 expression patterns**

Gene name	NCBI Accession Number	Primer Sequence	Amplicon size (bp)
HECW1	NM_015052.3	5'-AAAGACTCCTGGAGAGACACGC-3' 3'-GATGGAGGAAGTGATCTCAAATCGG-5'	122

A non-template control and an RNA sample not digested with DNAse were used each trial to control for potential contamination. All RT-qPCR reactions were performed in triplicate, and the resultant values were combined into an average cycle threshold (Ct). Real-time SYBR green fluorescence (PerfeCTa SYBR Green FastMix w/ low ROX, Quanta Biosciences) was measured using a 7500 Real Time PCR system (Applied Biosystems). The expression profiles for the mRNA transcripts were shown as the relative expression levels calculated by the ΔΔCt method as previously described (Schmittgen and Livak 2008). Values were normalized (ΔCt) to actin. One way analysis of variance (ANOVA) and Tukey's post hoc test ($p>0.05$) were used to determine gene expression levels and determine if there were any significant differences in levels of gene expression. RT-PCR was performed using the same primers as RT-qPCR for 30 cycles.

SDS-PAGE and Immunoblot

Cells from half of a 10 cm tissue culture dish from each transfected culture were lysed in 150µl cold lysis buffer (40mM Tris pH 7.5, 150mM NaCl, 5% glycerol, 0.01% Brij-35, 0.01% Triton X-100, 0.005% Tween 20) with protease inhibitor cocktail (Roche). Cells were lysed on ice using a Missonix XL-2000 ultrasonic cell disruptor by 3 x 10 pulses at power 2. Total cell lysate was mixed with 5X Laemli sample buffer. Samples were boiled for 5 minutes and separated electrophoretically on 12% SDS-PAGE gel, using a Bio-Rad mini protean system (Bio-Rad). For Western blot analysis, the proteins were transferred to PVDF membrane (Millipore) using a semi dry transfer apparatus (GE Healthcare). Membrane was blocked in TBST buffer (25mM Tris-HCl pH7.5, 130mM NaCl, 0.1% Tween-20) with 5% fat-free dry milk for 30 minutes. The membrane was incubated with mouse anti-His primary antibody overnight at $4^{o}C$. After washing the membrane 3 x 10 minutes in TBST buffer, the membrane was incubated in alkaline phosphatase-conjugated anti mouse secondary antibody for 2 hours at room temperature. The bands

were visualized using Western blue reagent (Promega, Fitchburg, WI). Images were acquired with a Canon LiDE 200 scanner.

Chromatin affinity precipitation and sequencing

HeLa cells expressing His-tagged FGF2 isoforms were cross-linked with 1% formaldehyde in PBS for 10 minutes at 30^{O}C. Cells were then lysed in buffer containing 20mM Tris-HCL pH 7.9, 500mM NaCl and EDTA-free protease inhibitor cocktail. Chromatin was fragmented by sonication to an average size of 1kb, and centrifuged at 16,000 x g for 10 minutes in a refrigerated centrifuge. The supernatant was diluted 5-fold in binding buffer (500 mM NaCl, 20 mM Tris-HCl, pH 7.9), then incubated in the 4^{O}C for 1 hour, shaking with 50µl salmon sperm DNA and 150 µl of pre-equilibrated nickel resin slurry (GE Healthcare). Resin was washed twice with buffer containing 50mM imidazole (J.T. Baker). Protein-DNA complexes were eluted with buffer containing 500mM imidazole, and cross-linking was reversed by incubation of protein-DNA complexes at 65^{O}C for 4 hours. RNA was digested using 100 units RNase (Macherey-Nagel) for 30 minutes at 37^{o}C, followed by protein digestion with 0.5 mg/ml proteinase K (New England Biolabs) at 55^{O}C for 1 hour. DNA was purified by phenol-chloroform extraction. Recovered nucleic acids were end-filled using DNA Polymerase I large (Klenow) Fragment (New England Biolabs) to generate blunt end inserts and cloned in Zero Blunt® Topo® PCR Cloning (Life Technologies) following the manufacturer's guidelines. DNA was transformed into chemically competent *E.coli*, plated on LB-agar plates containing 50µg/ml kanamycin and 25µg/ml zeocin, and incubated overnight at 37^{O}C. Single white colonies were picked and grown from each transformation. Plasmid DNA was purified using Macherey-Nagel plasmid miniprep kit, and sequenced using M13 forward and M13 reverse primers.

Electromobility shift assay (EMSA)

Positive clones identified from the sequencing results were isolated from the Zero Blunt® Topo® vector using EcoRI (New England Biolabs). Lower molecular weight bands corresponding to the cloned

DNA were separated on a 1% agarose gel, and were gel purified using a NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The DNA concentration was determined using nanodrop (Thermo Scientific). An EMSA reaction was set up using 1 µg of DNA, 10 ng of protein for each FGF2 isoform, 0.1 mM DTT, 5 µl 5X buffer (10 mM Hepes, pH 7.9, 50% glycerol, 50 mM KCl, 0.5 mM EDTA, 1.0mM dithiothreitol, protease inhibitor cocktail) in a 25 µl reaction. Bands were resolved on a 1% agarose gel in 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA) pre-run at 90V for 1 hour. The gel was run for 2 hours at 90V, and imaged using Bio Rad Gel Doc™ XR+ system (Bio Rad).

Results

FGF2 isoform sub-cloning and over-expression

All five individual FGF2 isoforms were isolated by restriction digestion (Figure 5.2 a) and directionally ligated into pLVX-Puro (Figure 5.2 b) as discussed in chapter 2.

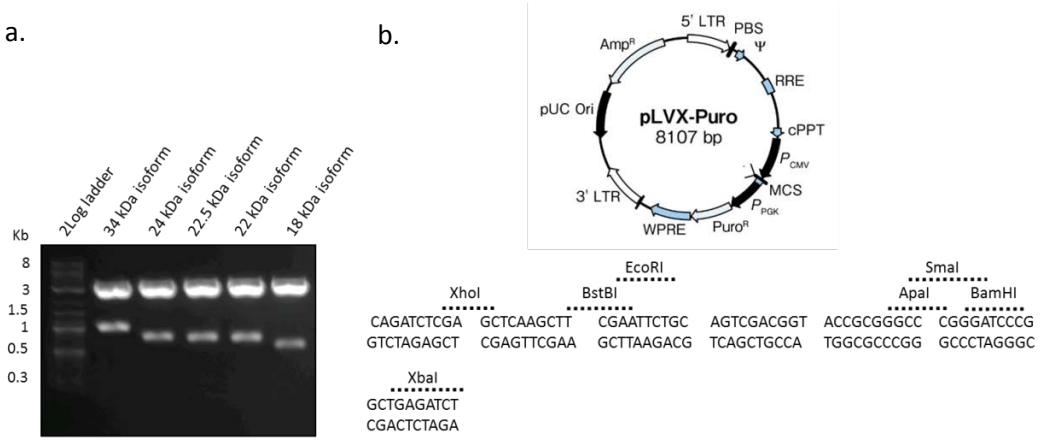

Figure 5.2 | **Isolation of recombinant FGF2 genes**
Gene inserts for all five FGF2 isoforms were isolated by restriction digestion followed by agarose gel purification from pBluescrip cloning vector (a). Individual isoforms were sub-cloned into the pLVX-Puro mammalian expression vector (b).

Transfections in HeLa cells were analyzed by RT-PCR and Western blot for gene and protein expression, respectively, 48 hours after transfection. Protein expression was confirmed for four of the five FGF2 isoforms, using mouse anti-His primary antibody, selectively binding to over-expressed tagged proteins followed by HRP conjugated anti-mouse secondary antibody (Figure 5.3). All bands visible on the Western blot migrated at the expected molecular weight for FGF2 isoforms.

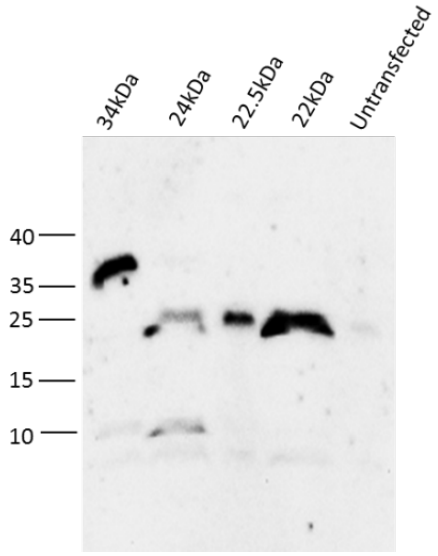

Figure 5.3 | **Western blot analysis of FGF2 isoform over-expression in HeLa cells** Proteins were detected with mouse anti-His primary antibody and visualized through HRP conjugated anti-mouse secondary antibody. Untransfected cells were used as a negative control.

Chromatin affinity precipitation and sequencing

All chromatin fragments isolated from the affinity precipitation using Ni-NTA resin were cloned into the pCR-Blunt II-TOPO vector. The presence of cloned chromatin fragments was analyzed by restriction digest with EcoRI restriction enzyme (Figure 5.4). The chromatin affinity precipitation followed by TOPO cloning resulted in one positive target (denoted by a white arrow in fig 5.4) isolated from the pull down of the 22.5kDa FGF2 isoform.

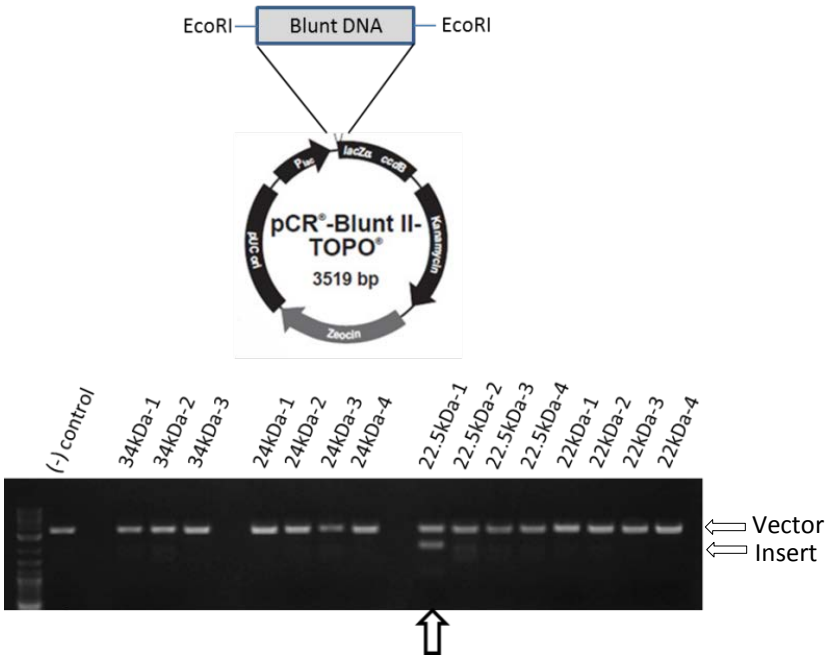

Figure 5.4 | **Agarose gel electrophoresis of EcoRI restriction digested DNA fragments cloned in PCR ZeroBlunt Topo vector** - DNA fragments isolated from the chromatin affinity precipitation (ChAP) assay were cloned in the ZeroBlunt Topo vector, and were then isolated by cleaving with EcoRI restriction enzyme. EcoRI digestion cleaves on both sides of the DNA insert. The numbers after the name on each lane indicates the clone isolated from the transformation.

Sequencing analysis returned positive results for one target (22.5_Clone #1) originally bound by the 22.5kDa FGF2 isoform (Figure 5.4). The target sequence identified is E3 ubiquitin protein ligase 1 (Figure 5.5) coded by the HECW1 gene located on chromosome 7.

HECW1 – E3 Ubiquitin protein ligase 1

>D31-M13R_A02.ab1
NNNNNNNNNNNNNNNNNNGNNNNNNNATANNNNACTCAAGCTATGCATCAAGGCTNTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAG
TGTGCTGGAATTCGCCCTTTGGTCTTCTGATATTTAACCAGTAAGGAAAAATTAATAGTACATATCAAACTTGTACCTACTAAACAGGAAGTACATCTTCC
TTGTAAATGGAACATGCTCAAATATATCAATATATTTGACAAACCACATCACTGGACCACAATGCAGAAATGTTAGAAATTATTAATAAAATTTTAGACAC
AGACATGTAAACACATTACTACTTGGAAAGATTGTTGACTATTGTAAATAAGTATTTTGCCATAGAGGAAATCAATACCACATTTATAGAACCTTAAGAAA
ATGGAGGGCAGTCATTCATTATTTGCAAATCCAAGAAATAATTTTAAAATACTATTAGAACTGACATGGAATTTGAGTAACAAGATTAAGGCTACATCTCCA
AAGAGAAATTGCTTTTCTAACAACTACCAAAAAAAAAAATTACAATCTTCAAAAAGTAAACATCTCATTTATCTCATCAACAAAAAGCATAAAATACACAG
GCATGTACCTAGAAGTGCCTTGTTCTAATAAAATAGTAAATATTATCTCAATCCTGTATGCTTCACATGAACTCTTTCTGATTCTCACAACTCCACAAGTGG
GGTTACTGTCTCCATGTTTACTATACAAAGCATTTCAACAATATATATAAAAAATTCCATTTTCCTCGGTCAGACCTCAGGAAAATGACGTCTGGACAACT
AAGTAAATTGTCAGATGCCACATAGCTCTTGGAGAGGCAGGCTGGAAACCTCTGTCCTTCTTCAAAGCCTCTTCAAAGTCTTTCCACTCTCCACTGTCC
CTTGATCAGCTGCACAACCAATACAAAGAAAACAGATGCTGACAAGAGGGCTCTGAACTGAATGTAGAGACATCTATGTCCCCAGATGGGAAAACTAG
ACATGAGAANACTTTTTTCCCCTTAAAATAATGTACGGGTNGAATGTAACTCCNACCAAAATCTCTATGANACTTCCTTTTGTAGGGGANNCACTTTGT
GNAANGNTACCCNNNNTTNNCGGNNNNTNCNTCGANNNGGAAGGGAANNCNNNNNCCCATNNNCCATTCNNCNTTT

Figure 5.5 | **Sequencing result of positive clone identified from chromatin affinity precipitation** M13 forward and M13 reverse sequencing primers on the vector were used. Underlined sequence indicates an FGF2 responsive element previously identified in the promoter of rat bone sialoprotein (Boudreaux and Towler 1996)

Electromobility shift assay (EMSA)

The binding of proteins to nuclear DNA sequences has been shown to be involved in the regulation of fundamental biochemical processes including the regulation of gene expression. To confirm our findings from the chromatin affinity purification and sequencing experiments we attempted to determine if FGF2 isoforms bind the identified DNA sequence *in vitro* using an EMSA. The DNA sequence identified as a potential FGF2 target was isolated from the pCR-Blunt II-TOPO vector using EcoRI restriction digestion and subjected to a gel retardation assay. Under the conditions tested, none of the FGF2 isoforms appeared to bind the DNA fragment identified to modify the electrophoretic profile on a 1% agarose gel in the expected size of 500bp (Figure 5.6)

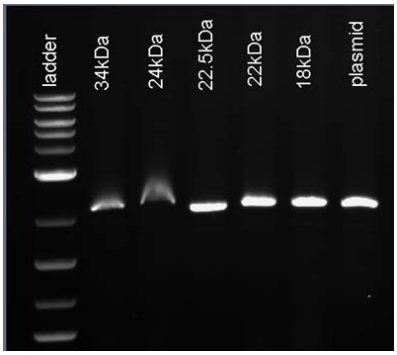

Figure 5.6 | **Electromobility shift assay of HECW1 DNA with FGF2 isoforms**
2-log ladder is loaded in the first lane. All FGF2 isoforms incubated with HECW1 DNA in EMSA binding buffer were loaded in lanes 2-6. Control HECW1 DNA alone, with no FGF2 protein was loaded in the last lane.

Effect of FGF2 isoforms on expression of HECW1 gene

To acertain the potential change in HECW1 gene expression, individual, and various FGF2 isoforms were over-expressed in adult human dermal fibroblasts CRL2352 (ATCC) through lipofectamine (Life Technologies) transfection. RT-PCR and RT-qPCR analysis were performed 36 hours post transfection on human dermal fibroblast cells expressing individual isoforms of recombinant FGF2, as well as control cells transfected with pLVX-Puro vector alone. HECW1 RT-PCR analysis shows gene expression in all transfected and untransfected cells (Figure 5.7) indicating no strong alteration of HECW1 expression by an FGF2 isoform relative to background levels in this assay, although the 34kDa isoform appears to decrease HECW1 expression relative to control.

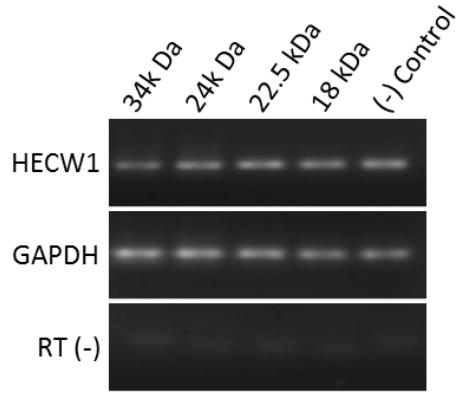

Figure 5.7 | **RT-PCR analysis of HECW1 expression in FGF2 transfected cells**
Primers indicated above were used for amplification of HECW1 GAPDH and RT (-) control of FGF2 isoform transfected human dermal fibroblasts. Cells transfected with pLVX-Puro vector alone are used as a negative control.

Quantitative RT-PCR analysis was performed on FGF2-isoform-transfected and pLVX-Puro transfected control human dermal fibroblasts to assay any potential differences in gene expression levels. The data suggests that all FGF2 isoforms have a transcriptional inhibition effect on HECW1 gene expression as compared to the pLVX-transfected control. Expression of HECW1 gene is down-regulated an average of 1-4 fold in cells expressing FGF2 isoforms (Figure 5.8).

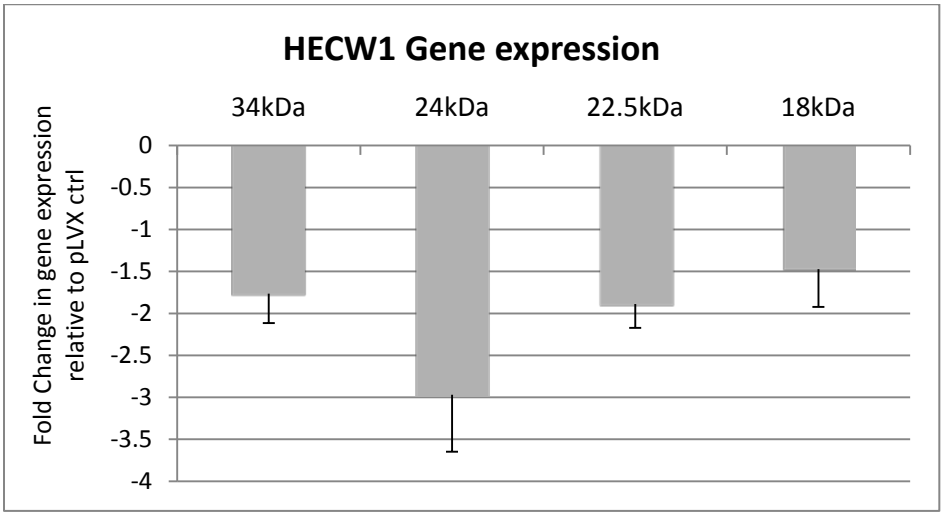

Figure 5.8 | **RT-qPCR analysis for HECW1 gene on FGF2 isoform transfected samples**
Data is analyzed using the ΔΔCT method. HECW1 expression is normalized to GAPDH and relative expression is determined as a comparison to pLVX-Puro transfected control. Error bars indicate standard deviation (SD)

Fluorescent imaging of human dermal fibroblasts transfected with a GFP expressing vector, using the same transfection method as for FGF2 variants, showed that only 10-20% of the cells express GFP 48 hours after transfection (Figure 5.9).

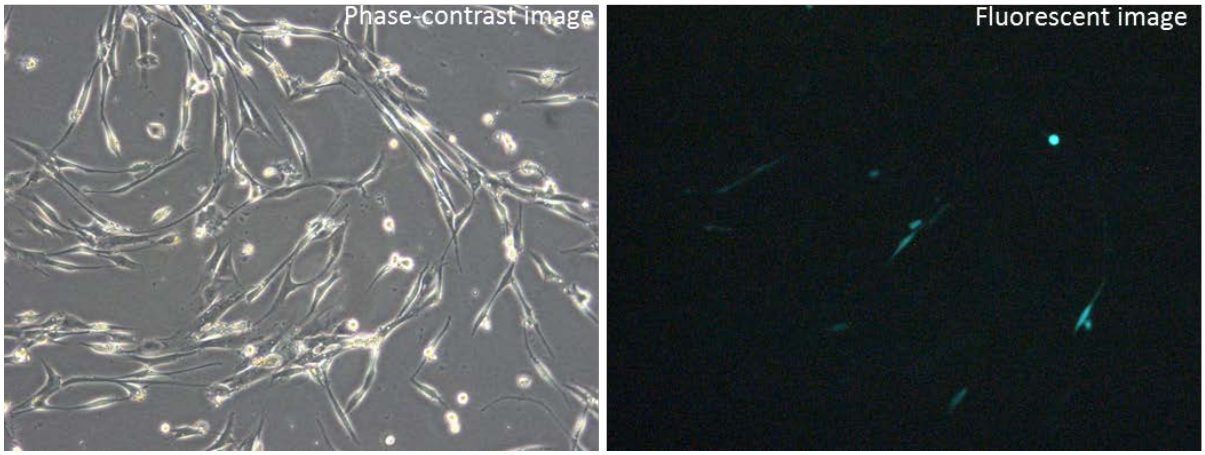

Figure 5.9 | **Lipofectamine 2000 transfection efficiency of adult primary human dermal fibroblasts**. Lipofectamine 2000 transfection of GFP-positive vector in primary human dermal fibroblasts yields 10-20% transfection efficiency 24 hours post transfection

Discussion

Identifying the nuclear targets of FGF2 isoforms can shed light on the pathways involving FGF2 in development and disease progression. Chromatin affinity precipitation experiments with Ni-NTA resin, targeting individual FGF2 isoforms fused to 6xHis tag identified a target gene bound by FGF2 or protein complexes involving FGF2 protein. The target gene, identified as HECW1, also known as NEDD4-like ubiquitin protein ligase 1 (NEDL1), is an E3 ubiquitin protein ligase that has been described to mediate the ubiquitination and subsequent degradation of disheveled-1 (DVL1) and superoxide dismutase-1 (SOD1) proteins leading to motor neuron death in familial amyotrophic lateral sclerosis (FALS) (Miyazaki et al., 2004). Careful analysis of sequencing results, and comparison of those results with previously published data, identified the presence of a 7 base-pair stretch included in the identified sequence (Figure 5.5) that has been previously described to be an FGF2 response element (FRE), recognized and bound by FGF2 (Boudreaux and Towler 1996; Takai et al., 2008). The FRE was first

identified in the rat bone sialoprotein (BSP) gene promoter, and binding of FGF2 to the FRE was shown to induce BSP transcription (Shimizu-Sasaki et al., 2003). An electromobility shift assay was done using the DNA sequence identified from the chromatin affinity precipitation with over-expressed and purified recombinant FGF2 isoforms but no shift was detected for any of the FGF2 isoforms (Figure 5.6). This could be due to reaction binding conditions not offering optimal protein-DNA complex stability, or reaction conditions not being suitable for binding. The negative outcome of the EMSA assay can also be a result of lack of other proteins necessary to facilitate the binding of FGF2 proteins to the DNA target. The cell lysate might contain a mixture of proteins and protein complexes that are lacking in the *in vitro* EMSA assay. It is well described in the literature that many proteins form protein-DNA complexes only when interacting to form heteromeric protein complexes (Aoki et al., 2012; Maity et al., 1992; Thompson et al., 1991). Following the sequencing and EMSA results, we were interested to investigate if over-expression of individual FGF2 isoforms had an effect on the expression of the HECW1 gene. Traditional RT-PCR analysis revealed the presence of the HECW1 mRNA in cells expressing recombinant FGF2 isoforms but it was also present in the cells transfected with just pLVX-Puro vector. Overexpression of the 34kDa appeared to decrease HECW1 transcript levels. To determine quantitatively if there was a difference in gene expression level between cells expressing recombinant FGF2 isoforms and the ones not expressing recombinant FGF2, RT-qPCR was done. Relative quantification of expression showed a 1-4 fold difference in expression of the HECW1 gene for populations of cells expressing recombinant FGF2 isoforms. We believe the difference in relative expression on a per cell basis to be higher than the difference at the population level as transfection efficiency of lipofectamine in primary human dermal fibroblasts is only 10-20% (Figure 5.9). E3 protein ubiquitin ligase NEDL1 is also known to regulate the function of tumor suppressor p53 in an enzymatic independent manner. NEDL1 binds to the COOH-terminal of wild type p53 and induces apoptosis in neuroblastoma SH-SY5Y cells (Li et al., 2008). In the future we propose to expand the RT-qPCR experiments to determine the role of FGF2 isoforms on

affecting p53 expression and activity. p53 is an important tumor suppressor whose function is disrupted in many cancers. Regulation of p53 expression and function in an FGF2-dependent manner may explain involvement of FGF2 isoforms in increased cells' life span and its role in cancer progression.

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Conclusions:

The initial body of work presented in this thesis shows the potential of specific *Xenopus laevis* egg extract fractions to reprogram differentiated cells into a multipotent state. Furthermore, this work shows the importance of exogenously added FGF2 as an absolutely required factor for reprogramming human dermal fibroblasts, and to maintain multipotency in the reprogrammed cells. In an effort to identify the specific role of individual FGF2 isoforms in cellular reprogramming events, we show that all HMW FGF2 isoforms can support the growth of human pluripotent stem cells in an undifferentiated state, and demonstrate that FGF2 isoforms have the potential to induce the expression of stem-cell specific proteins in human dermal fibroblasts and extend their lifespan *in vitro*. Moreover, we identified a novel FGF2 nuclear target by ChIP and RT-qPCR. E3 ubiquitin ligase HECW1 was shown to be a target gene for all FGF2 isoforms and the transcript level of this gene is decreased as a result of FGF2 isoform over-expression.

Here we report the potential of select *Xenopus laevis* egg extract fractions, in combination with exogenous FGF2, to affect life span, morphology, gene expression, protein translation and cellular localization of OCT4/NANOG/SOX2 transcription factors, and developmental potential of human dermal fibroblasts *in vitro*. The loss of GFP tagged Histone H1.2 protein in extract-fraction treated cells suggests that these cells were undergoing chromatin decondensation and nuclear reprogramming. Screening of active egg extract fractions for nuclear reprogramming factors revealed the presence of nucleoplasmin, maskin, FGF2 and TGFβ1 proteins. RT-PCR analysis for gene expression levels in treated and untreated human dermal fibroblasts did not demonstrate major differences in mRNA expression. However, the translation of stem cell associated mRNA was significantly affected by the extract in combination with exogenously added FGF2**.** The translation of embryonic transcription factors and their nuclear localization was accompanied by a gradual change in cell morphology and expanded life span exceeding 60 population doublings. Immunocytochemistry and Western blotting detected the presence of OCT4

and NANOG proteins in treated cells and the transcription factors localized to the cells' nuclei. The potential of the treated cells to follow ectodermal tissue lineage development was indicated by the acquisition of a neuroepithelial phenotype and the expression of nestin and Tuj1, both markers of neuronal differentiation. The deposition of calcium in electroporated cells exposed to osteogenic conditions was detected with Alizarin Red. Under conditions that promote adipogenic differentiation, cells began accumulating lipids. Injection of reprogrammed cells in SCID mice did not result in formation of teratomas, which demonstrates that these cells have not acquired full pluripotency.

Chapter 2 conclusions

The natural reprogramming activity of egg cytoplasm without the need for transcription may lead to identification of the translational stimulatory molecules and mechanisms of nuclear reprogramming, and consequently the use of these molecules to enhance the efficiency of reprogramming. This system could be applied for derivation of therapeutically relevant, patient-specific multipotent cell types. This would be of interest especially if the reprogrammed cells of one type could yield cells of another therapeutically relevant cell-type, rather than being pluripotent and potentially tumorigenic.

Recently, Awe and Byrne identified eight candidate oocyte reprogramming factors (CORFs) (Awe and Byrne 2013) that closely correlate with the "chromatin loosening/enhanced reprogramming" hypothesis previously described by Gurdon and Wilmut. Five of those eight CORFs (ARID2, ASF1A, ASF1B, H1FOO and KDM6B) not only are expressed in *Xenopus laevis* oocytes but their molecular weights fit the S200 elution profile in our study, suggesting that these factors are likely present in the pools of fractions that can reconstitute reprogramming activity of the total extract. While FGF2 has not been implicated previously in the transcriptional activation of OCT4 or SOX2, it has been determined that the maintenance of expression of these genes and cell pluripotency is dependent on FGF2 (Levenstein, Ludwig et al. 2006). The proposed action of FGF2 involves induction of members of TGF-β

pathway; TGF-β ligands maintain expression of OCT4, SOX2, and NANOG which in turn activate expression of endogenous FGF2 that completes this regulatory loop (Greber, Lehrach et al. 2007). It is reasonable to hypothesize that similar FGF2-dependent core regulatory circuit maintaining the self-renewal ability and maintenance of pluripotent state in embryonic stem cells may at least in part be needed for reprogramming of differentiated somatic cells.

Chapter 3 conclusions

We analyzed the biological activity and reprogramming potential of five recombinant FGF2 isoforms on adult human dermal fibroblasts and human ESCs. We established an expression and purification process that allowed for production of highly pure, biologically active recombinant FGF2 isoforms. All five FGF2 isoforms were individually synthesized fused with a 6xHis-tag and a tobacco etch virus (TEV) cleavage site. All five FGF2 isoforms ranging from 18kDa to 34kDa were over-expressed as individual isoforms using bac-to-bac® expression system, and coomassie stained SDS-PAGE followed by Western blot analysis indicated that individual isoforms can be isolated through a single Ni-NTA chromatographic step with up to 85-90% purity. Although the coomassie staining showed the presence of a few minor contaminating bands, they are unlikely to be responsible for the activities observed in this study. To test biological activity of all individual FGF2 isoforms we monitored the ability of each isoform to induce phosphorylation of FGF receptor 1, phosphorylate FRS2α, and activate the mitogenic MAPK pathway by inducing phosphorylation of ERK1/2 in adult human dermal fibroblasts. All recombinant HMW FGF2 isoforms, exogenously supplemented in the culture medium, had the ability to phosphorylate FGF receptor 1, phosphorylated FRS2α, as well as activate the mitogenic pathway through ERK1/2 phosphorylation. Semi quantitative analysis showed that activity levels of all recombinant FGF2 isoforms purified in our lab are similar to the activity of the commercially available 18kDa isoform. All FGF2 isoforms had the ability to maintain the expression of stem cell specific mRNAs,

stem cell specific proteins as well as support undifferentiated growth of human embryonic stem cells on inactivated mouse embryonic fibroblasts for at least 28 days.

Treatment of adult human dermal fibroblasts with individual FGF2 isoforms induces the expression of stem cell specific proteins and extends the lifespan of these cells as compared with untreated controls. All five FGF2 isoforms can be over-expressed as recombinant proteins and can be purified with high purity through a single chromatographic step. To our knowledge, this is the first study to analyze the ability of individual HMW FGF2 isoforms to activate signal transduction of the MAPK canonical pathway when exogenously supplemented in the culture medium of human dermal fibroblasts *in vitro*. This is also the first study to analyze the ability of HMW FGF2 isoforms to support expansion of human embryonic stem cells in an undifferentiated state. We conclude that the secreted HMW FGF2 isoforms have biological activity that mimics the activity of the LMW secreted FGF2, and may be important in signaling.

Chapter 4 conclusions

We compared the biological activity of recombinant FGF2 isoforms with commercially available 18kDa FGF2 on human dermal fibroblasts and human pluripotent stem cells *in vitro*. We systematically determined the mitogenic activity of each FGF2 isoform interacting with specific recombinant FGF receptor variants expressed in BaF3 cells. A direct comparison of the mitogenic activity of each individual FGF2 isoform with single FGF receptor variants showed that all FGF2 isoforms display the highest level of relative mitogenic activity when interacting with receptor FGFR1 IIIb. The data also shows that the relative mitogenic activity for HMW weight isoforms is lower than activity of 18kDa isoform and FGF2 mitogenic activity for all FGF2 isoforms reacting with FGFR3 IIIb is very low. The findings from this work support the hypothesis that HMW FGF2 isoforms are released from cells in the extracellular space to potentiate 18kDa mitogenic activity when higher levels of secreted ligand are demanded by the cell population.

Chapter 5 conclusions

Cell lysate from transgenic HeLa cells expressing 6xHis tagged recombinant FGF2 isoforms were subjected to chromatin affinity precipitation (CHAP), and nucleic acids pulled down were sequenced for each specific over-expressed isoform. The HECW1 gene coding for E3 ubiquitin ligase NEDDL1, was identified as a positive target from the CHAP assay. The HECW1 gene contains a 6 basepair DNA sequence that has been previously described as a FGF2 response element (FRE) (Takai, Araki et al. 2008). The follow-up EMSA returned negative results and did not however confirm HECW1 as a gene target for any of the FGF2 isoforms and may indicate unfavorable binding conditions *in vitro*, which do not create an appropriate environment for protein – DNA binding or alternatively the negative EMSA outcome may also suggest that other proteins are required that facilitate binding of FGF2 isoforms to the DNA sequence on this specific gene target. Additional RT-qPCR experiments confirmed a down regulation of HECW1 upon forced expression of FGF2 isoforms. RT-qPCR analysis for HECW1 gene expression levels showed that over-expression of four tested FGF2 isoforms in human dermal fibroblasts down-regulates HECW1 expression 1.5-3 fold, compared to vector transfected control cells.

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Future Work:

The ultimate goal of cellular reprogramming is to create off the shelf multipotent/pluripotent cells that can be used for personalized medicine, organ replacement therapies, disease modeling and drug testing. So far, a handful of methods have been described that allow for the generation of multipotent cells (Kim et al., 2009; Tada et al., 2001; Takahashi et al., 2007; Takahashi and Yamanaka 2006) but these methods are not yet safe and effective to be incorporated in personalized medicine (Ben-Porath et al., 2008; Kuttler and Mai 2006; Okita et al., 2007). There is still a need for reprogramming methods that will allow for the quick generation of safe, clinically relevant multipotent/pluripotent cells. Analysis of the isolated active extract fractions in our study revealed that, *Xenopus laevis* egg extract proteins and RNAs, as well as exogenously supplemented fibroblast growth factor 2, were required and sufficient for the induction and maintenance of this phenotypic change. Factors so far identified by Western blot in the active fractions include FGF2, TGFβ-1, maskin, nucleoplasmin and oocyte nuclear protein. However, we did not identify the minimal number of absolutely required factors that can reprogram differentiated cells. In order to determine a defined protein cocktail from *Xenopus* egg extract that can be used for the full reprogramming of differentiated cells, it is necessary to identify the minimal number of absolutely required reprogramming factors present in the extract. Establishment of a completely defined reprogramming system will require an investigation of mRNAs and micro RNAs from *Xenopus laevis* cytoplasmic egg extract. Future work will determine if *Xenopus* egg extract RNAs translate into protein when electroporated into human dermal fibroblasts, and will determine if these proteins are required for reprogramming human dermal fibroblasts in a multipotent state. The identification of all individual factors responsible for the induction of multipotency in human dermal fibroblasts will allow the establishment of a defined reprogramming system that can generate potentially clinically safe autologous multipotent cells.

Only a handful of FGF2 intracellular gene targets have been described so far anti-apoptotic protein Api1 (Krejci et al., 2007), ribosomal protein L6/TAXREB107 (Klein et al., 1996; Renko et al., 1990), splicing factor SF3a66 (Gringel et al., 2004), survival of motor neurons protein (Claus et al., 2003) promoter region of bone sialoprotein (Boudreaux and Towler 1996; Shen et al., 1998; Sheng et al., 2005; Shimizu-Sasaki et al., 2003; Takai et al., 2008). Work presented in this thesis identified HECW1 as a target gene for FGF2 isoforms. Based on previous publications that demonstrated NEDL1 can bind to p53 and can affect p53 activity in a ubiquitin independent manner (Li et al., 2008), we hypothesize that there may be a link between FGF2 isoform expression and p53 activity that affects the lifespan of human dermal fibroblasts *in vitro*. FGF2 isoform expression and translocation to the nucleus would down regulate HECW1 gene expression, which would decrease activity of tumor suppressor p53. Findings from other work in our lab have shown that exogenously added FGF2 has a similar effect and can down-regulate expression of HECW1 gene *in vitro* as well (Kashpur et al., 2013). To determine if FGF2 isoform expression affects p53 activity in a NEDL1 mediated manner, it will be necessary to determine the effect of individual FGF2 isoforms on NEDL1 protein expression. It is important to demonstrate if the down regulation observed for HECW1 mRNA translates to a down regulation at the protein level. Determining if overexpression of FGF2 has an effect on p53 activity through NEDL1 may open the doors to findings explaining the extended lifespan phenotype observed in human dermal fibroblast. Moreover with p53 being an important cancer target down-regulation of HECW1 resulting in reduced p53 activity may offer new knowledge that would help us understand the involvement of FGF2 in cancer progression.

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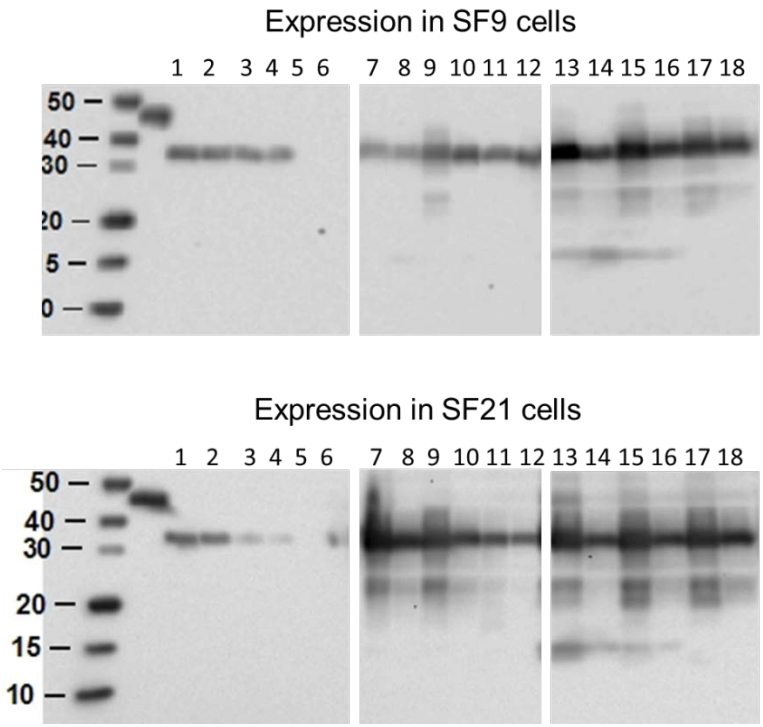
Introduction

As discussed in chapter 3, to determine best expression conditions that would yield highest protein expression with the lowest amount of protein degradation, a small scale expression optimization was performed in two different cells lines (SF9 and SF21), three different time points (24 hours, 48 hours and 72 hours) as well as three different viral dilutions (1:100, 1:1000 and 1:10000).

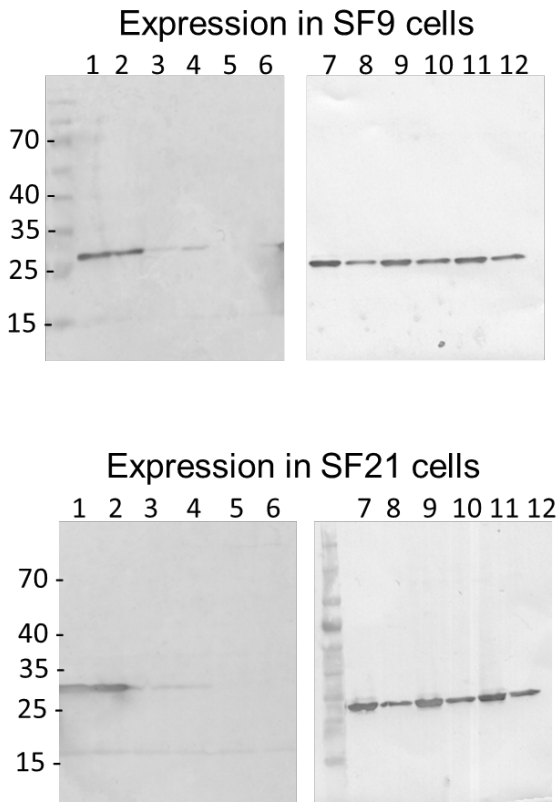
Determination of Best Expression Conditions

Western blot analysis was performed for cell pellets harvested at every indicated time point. Cells were rinsed once in PBS then lysed by sonication. Whole cell lysate was sampled in 2X Laemli gel loading buffer and soluble fraction was generated by centrifuging the samples at 10,000 x g for 15 minutes. Ten µL of each sample was resolved on a 4-20% SDS-PAGE gel and was then transferred on PVDF membrane. Western blots with anti-His specific primary antibody, followed by incubation in AP-conjugated anti-mouse secondary antibody were performed to visualize bands of interest (Figures A1 – A5).



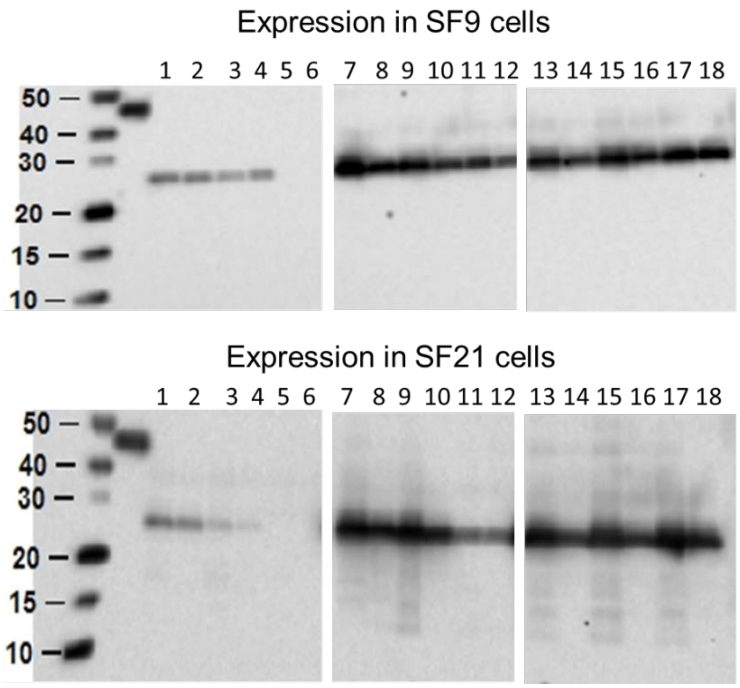
Lane	Viral Dilution	Sample loaded
1	1:100	24 hours whole cell
2	1:100	24 hours soluble
3	1:1000	24 hours whole cell
4	1:1000	24 hours soluble
5	1:10000	24 hours whole cell
6	1:10000	24 hours soluble
7	1:100	48 hours whole cell
8	1:100	48 hours soluble
9	1:1000	48 hours whole cell
10	1:1000	48 hours soluble
11	1:10000	48 hours whole cell
12	1:10000	48 hours soluble
13	1:100	72 hours whole cell
14	1:100	72 hours soluble
15	1:1000	72 hours whole cell
16	1:1000	72 hours soluble
17	1:10000	72 hours whole cell
18	1:10000	72 hours soluble

Figure A1 | **Western blot analysis of small scale expression optimization of 34kDa FGF2 isoforms in insect cells**. Table shows loading order and expression conditions for each sample loaded on the gel



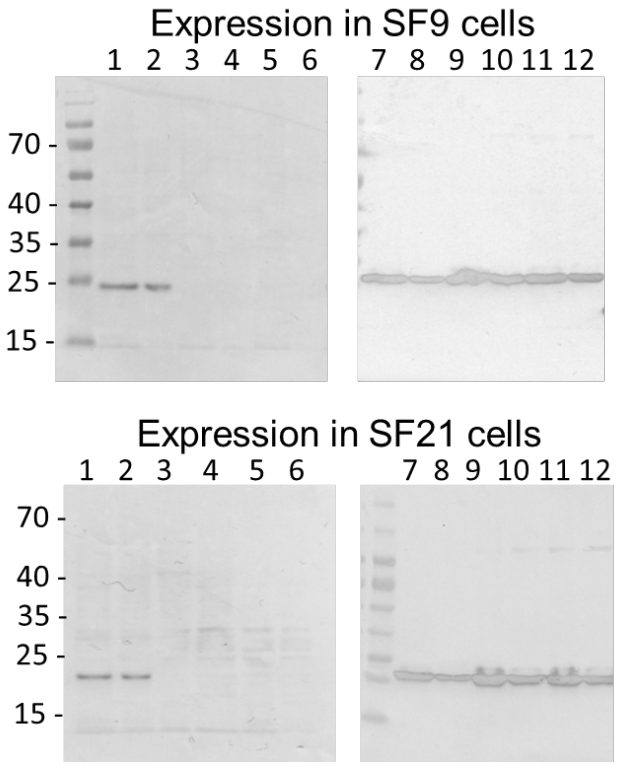
Lane	Viral Dilution	Sample loaded
1	1:100	48 hours whole cell
2	1:100	48 hours soluble
3	1:1000	48 hours whole cell
4	1:1000	48 hours soluble
5	1:10000	48 hours whole cell
6	1:10000	48 hours soluble
7	1:100	72 hours whole cell
8	1:100	72 hours soluble
9	1:1000	72 hours whole cell
10	1:1000	72 hours soluble
11	1:10000	72 hours whole cell
12	1:10000	72 hours soluble

Figure A2 | **Western blot analysis of small scale expression optimization of 24kDa FGF2 isoforms in insect cells**. Table shows loading order and expression conditions for each sample loaded on the gel



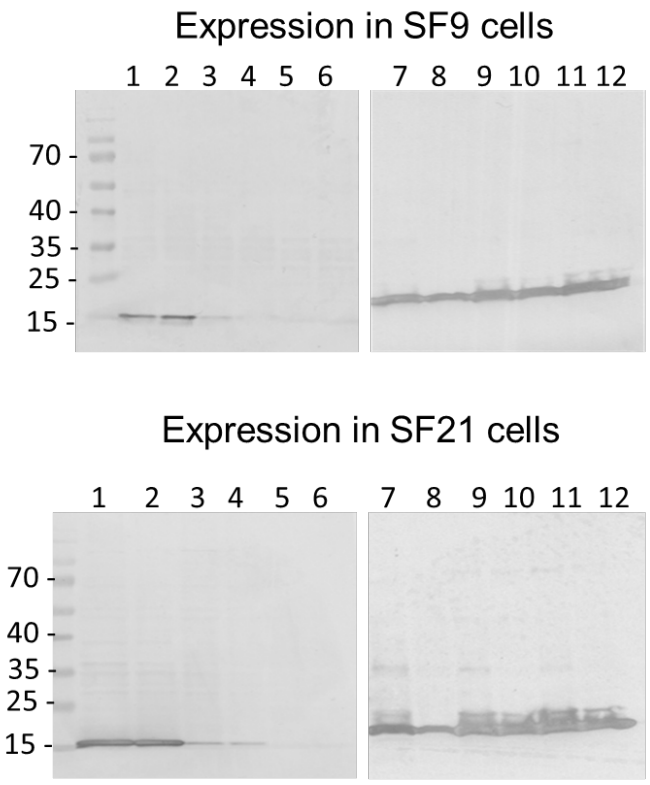
Lane	Viral Dilution	Sample loaded
1	1:100	24 hours whole cell
2	1:100	24 hours soluble
3	1:1000	24 hours whole cell
4	1:1000	24 hours soluble
5	1:10000	24 hours whole cell
6	1:10000	24 hours soluble
7	1:100	48 hours whole cell
8	1:100	48 hours soluble
9	1:1000	48 hours whole cell
10	1:1000	48 hours soluble
11	1:10000	48 hours whole cell
12	1:10000	48 hours soluble
13	1:100	72 hours whole cell
14	1:100	72 hours soluble
15	1:1000	72 hours whole cell
16	1:1000	72 hours soluble
17	1:10000	72 hours whole cell
18	1:10000	72 hours soluble

Figure A3 | **Western blot analysis of small scale expression optimization of 22.5kDa FGF2 isoforms in insect cells**. Table shows loading order and expression conditions for each sample loaded on the gel



Lane	Viral Dilution	Sample loaded
1	1:100	48 hours whole cell
2	1:100	48 hours soluble
3	1:1000	48 hours whole cell
4	1:1000	48 hours soluble
5	1:10000	48 hours whole cell
6	1:10000	48 hours soluble
7	1:100	72 hours whole cell
8	1:100	72 hours soluble
9	1:1000	72 hours whole cell
10	1:1000	72 hours soluble
11	1:10000	72 hours whole cell
12	1:10000	72 hours soluble

Figure A4 | **Western blot analysis of small scale expression optimization of 22kDa FGF2 isoforms in insect cells**. Table shows loading order and expression conditions for each sample loaded on the gel



Lane	Viral Dilution	Sample loaded
1	1:100	48 hours whole cell
2	1:100	48 hours soluble
3	1:1000	48 hours whole cell
4	1:1000	48 hours soluble
5	1:10000	48 hours whole cell
6	1:10000	48 hours soluble
7	1:100	72 hours whole cell
8	1:100	72 hours soluble
9	1:1000	72 hours whole cell
10	1:1000	72 hours soluble
11	1:10000	72 hours whole cell
12	1:10000	72 hours soluble

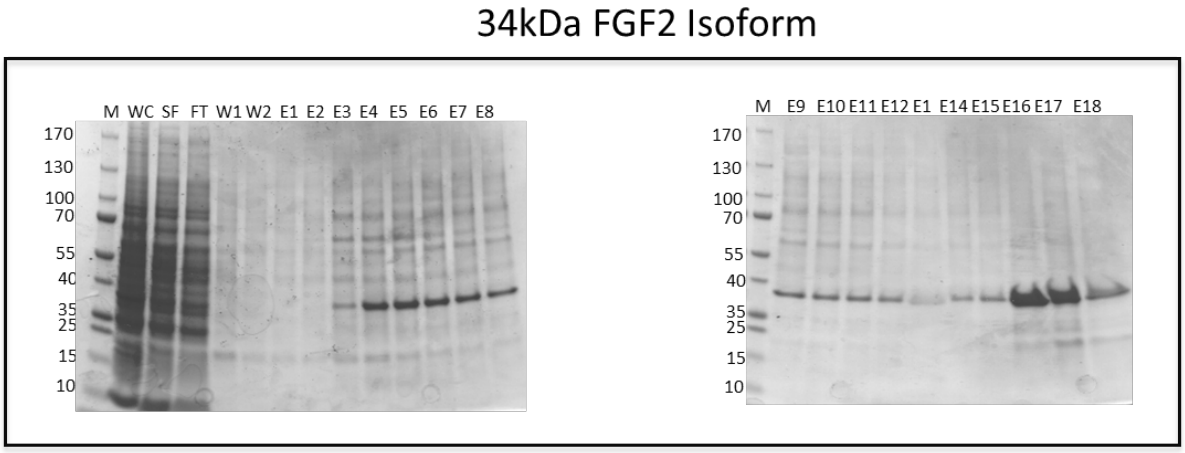
Figure A5 | **Western blot analysis of small scale expression optimization of 18kDa FGF2 isoforms in insect cells**. Table shows loading order and expression conditions for each sample loaded on the gel

Introduction

Generation of a highly pure preparation is very important in determining specific biologic activity of proteins. As described in chapter 3, all FGF2 isoforms were subjected to IMAC purification using an AKTA Explorer FPLC. Purification from each isoform was performed from the biomass generated from expression in 1L of virally infected SF21 cells. Low imidazole washes were performed to remove impurities and non-specifically bound proteins. Elution fractions at higher imidazole concentration were collected to determine purity of each FGF2 isoform.

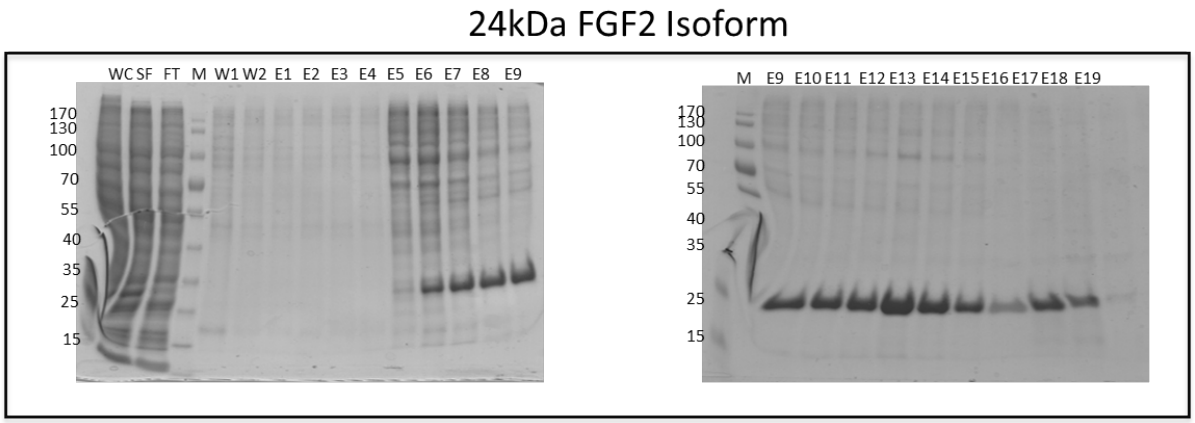
Identification of high purity fractions

Coomassie stained SDS-PAGE analysis was performed on the starting material, flow through fraction, wash fractions and all imidazole elution fractions. Each sample analyzed was mixed with 2X Laemli gel loading buffer and 10µl of each sample were loaded per well of a 4-20% SDS-PAGE gel. Gel were stained with coomassie brilliant blue stain and allowed to destain for a few hours prior to imaging (Figures B1 – B5). Fractions demonstrating >85% purity were pooled together for further analysis.



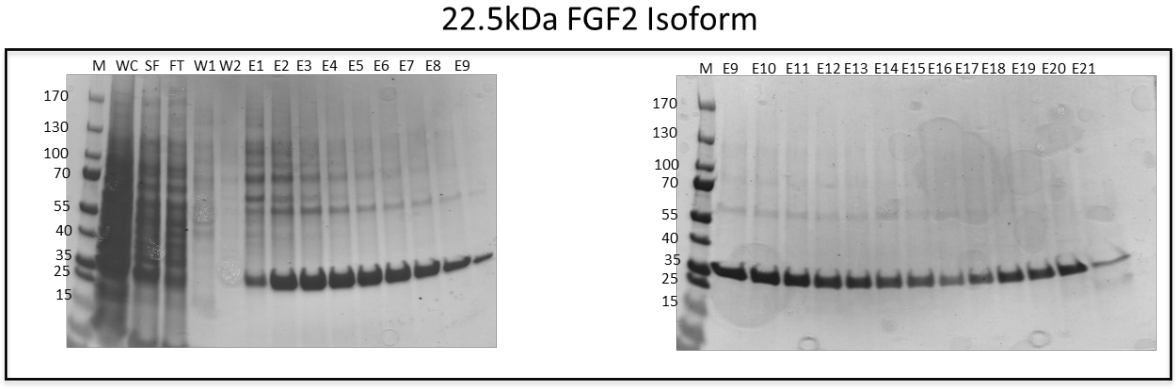
M - Protein ladder
WC – Whole cell lysate
SF – Soluble fraction (load)
FT – Flow through (unbound)
W – Washes
E – Elution fractions

Figure B1 | **Commassie stained SDS-PAGE gel of fractions from IMAC purification of 34kDa FGF2 isoform**.



M - Protein ladder
WC – Whole cell lysate
SF – Soluble fraction (load)
FT – Flow through (unbound)
W – Washes
E – Elution fractions

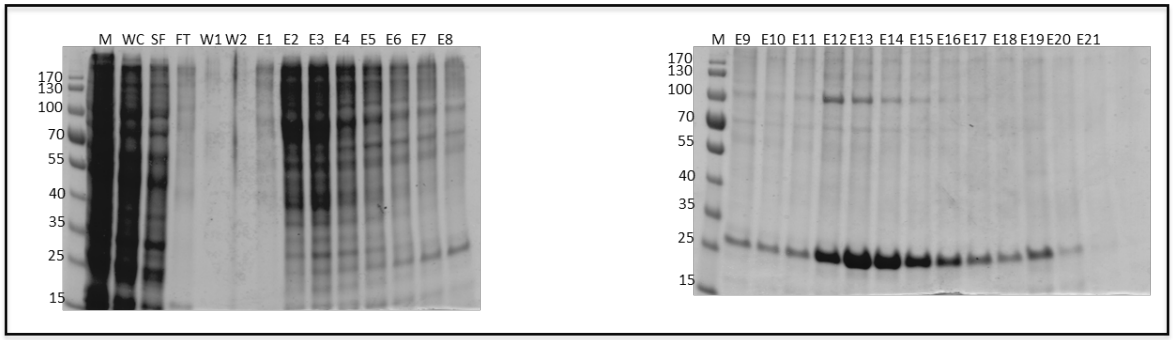
Figure B2 | Coomassie stained SDS-PAGE gel of fractions from IMAC purification of 24kDa FGF2 isoform.



M - Protein ladder
WC – Whole cell lysate
SF – Soluble fraction (load)
FT – Flow through (unbound)
W – Washes
E – Elution fractions

Figure B3 | Coomassie stained SDS-PAGE gel of fractions from IMAC purification of 22.5kDa FGF2 isoform.

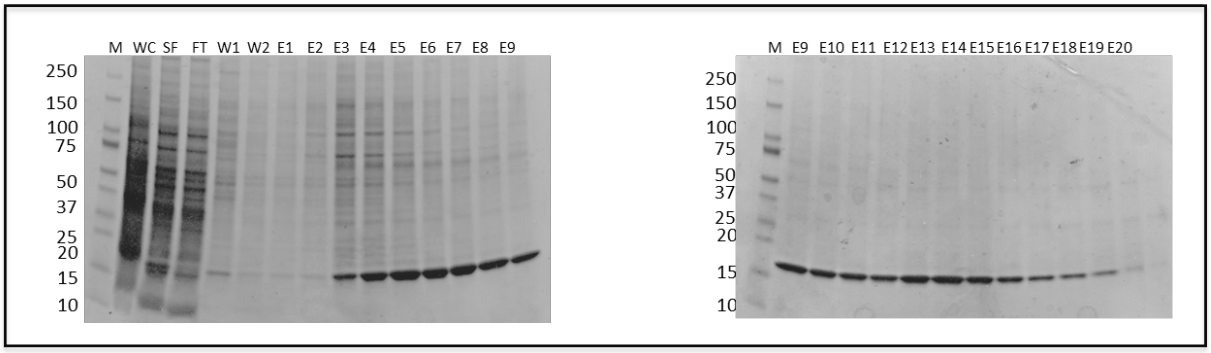
22kDa FGF2 Isoform



M - Protein ladder
WC – Whole cell lysate
SF – Soluble fraction (load)
FT – Flow through (unbound)
W – Washes
E – Elution fractions

Figure B4 | Coomassie stained SDS-PAGE gel of fractions from IMAC purification of 22kDa FGF2 isoform.

18kDa FGF2 Isoform



M - Protein ladder
WC – Whole cell lysate
SF – Soluble fraction (load)
FT – Flow through (unbound)
W – Washes
E – Elution fractions

Figure B5 | Coomassie stained SDS-PAGE gel of fractions from IMAC purification of 18kDa FGF2 isoform.

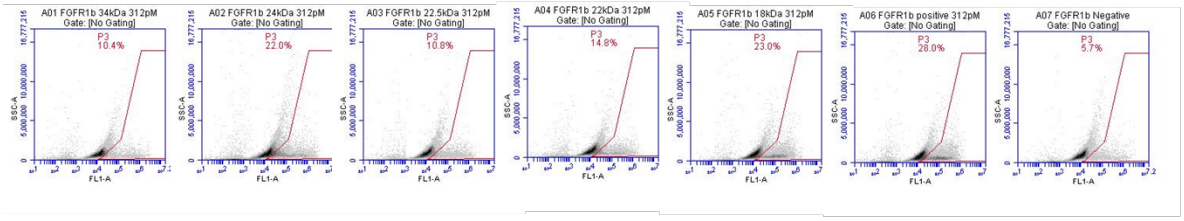
Introduction

As discussed in chapter 4, flow cytometry analysis for BrdU incorporation in BaF3 cells was performed to determine number of cells progressing into S-phase and correlate that with mitogenic activity of FGF2 isoforms when introduced to cells expressing select FGF receptor variants. Three receptor variants were tested; FGFR1 IIIb, FGFR2 IIIb and FGFR3 IIIb respectively, with three different concentrations for each FGF2 isoforms.

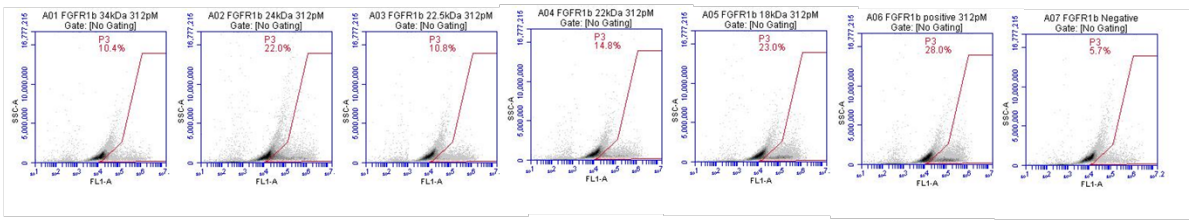
Mitogenic activity of FGF2 isoforms

Transgenic BaF3 cells expressing select FGF receptor variants were treated with different amounts of FGF2 isoforms and were then exposed to BrdU. Six hours after BrdU addition cells were rinsed, fixed and stained with fluorescently labeled anti-BrdU. Flow cutometry analysis using Accuri C6 cytometer was done to determine fractions of cells progressing into S-phase and therefore responding to FGF2 isoform treatments (Figure C1, C2, C3).

FGFR1b – 312pM FGF2 isoforms



FGFR2b – 312pM FGF2 isoforms



FGFR3b – 312pM FGF2 isoforms

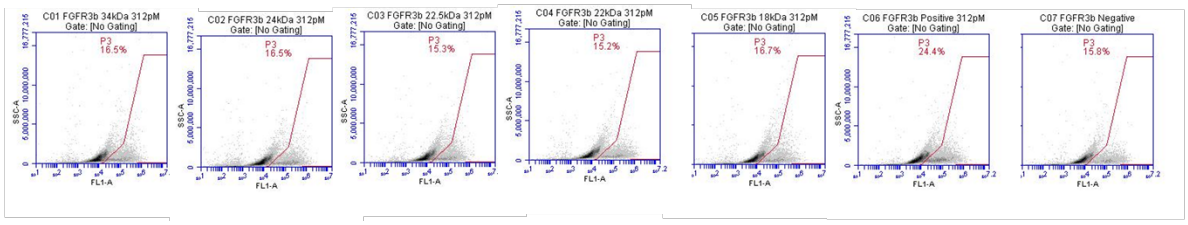
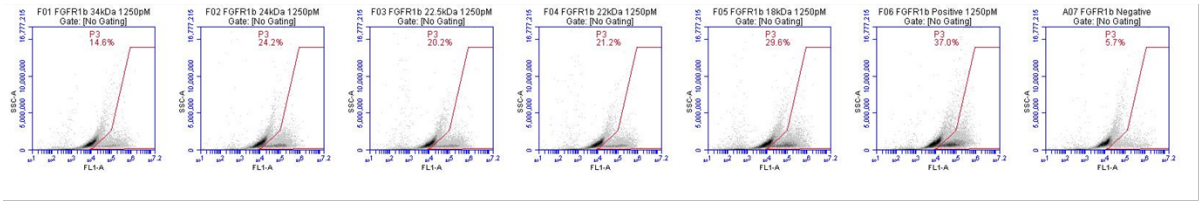
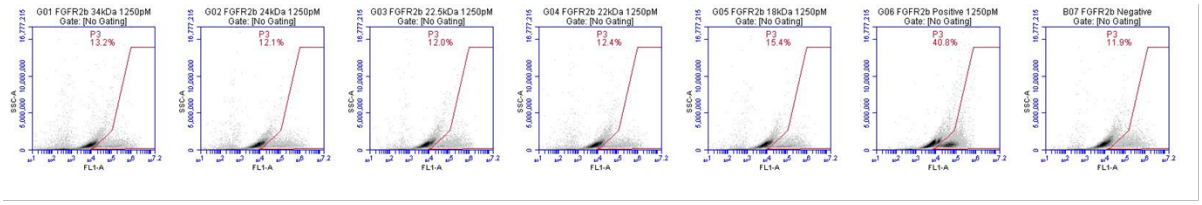


Figure C1 | **Flow cytometry analysis for BrdU incorporation at 312pM ligand concentration** 312pM of each FGF2 isoform was used to determine relative mitogenic activity of each isoform with individual FGFR variants. FGFR1 IIIb, FGFR2 IIIb and FGFR3 IIIb were tested. X-axis indicates fluorescence corresponding to cells stained positively with anti-BrdU antibody. Numbers for each graph indicate percentage of cells incorporating BrdU.

FGFR1b – 1250pM FGF2 isoforms



FGFR2b – 1250pM FGF2 isoforms



FGFR3b – 1250pM FGF2 isoforms

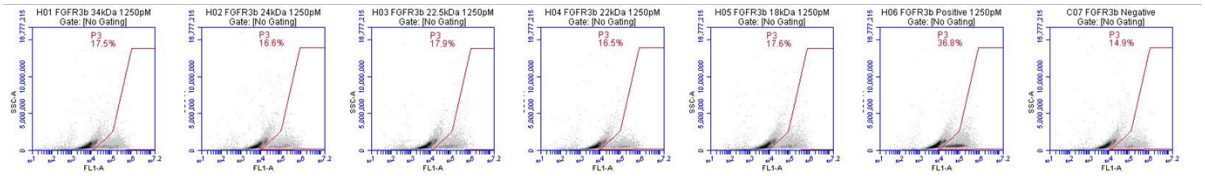
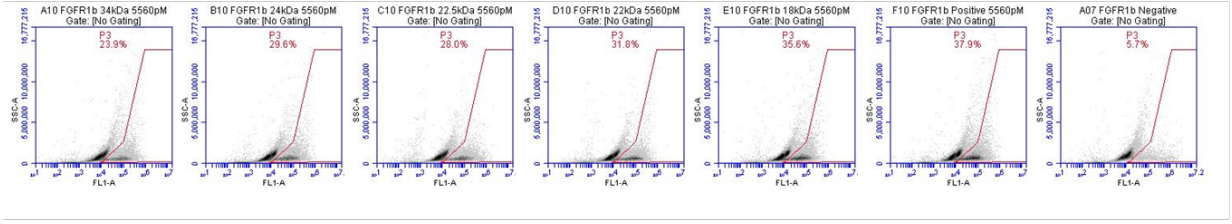
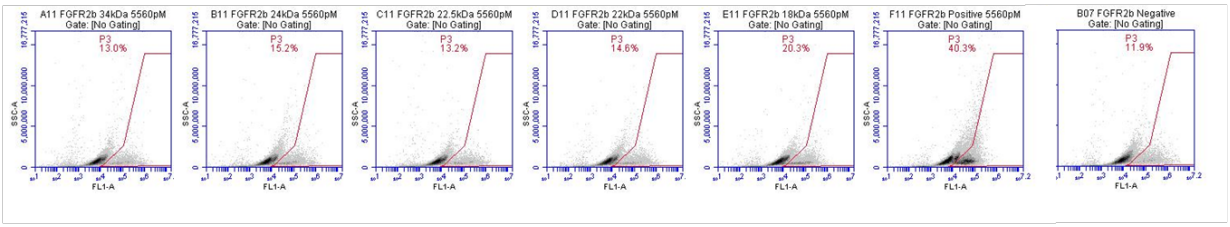


Figure C2 | **Flow cytometry analysis for BrdU incorporation at 1250pM ligand concentration** 1250pM of each FGF2 isoform was used to determine relative mitogenic activity of each isoform with individual FGFR variants. FGFR1 IIIb, FGFR2 IIIb and FGFR3 IIIb were tested. X-axis indicates fluorescence corresponding to cells stained positively with anti-BrdU antibody. Numbers for each graph indicate percentage of cells incorporating BrdU.

FGFR1b – 5560pM FGF2 isoforms



FGFR2b – 5560pM FGF2 isoforms



FGFR3b – 5560pM FGF2 isoforms

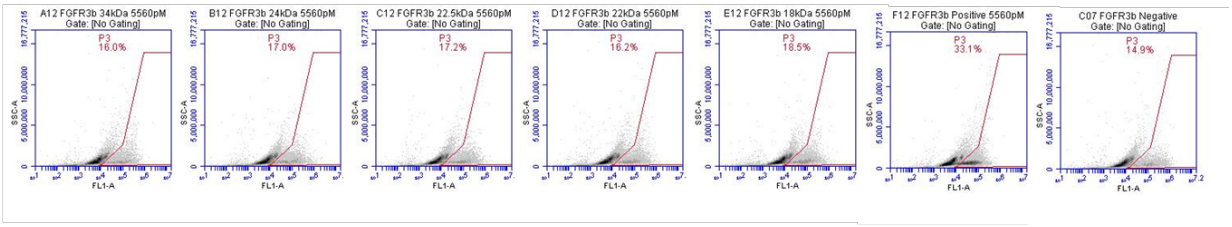


Figure C3 | **Flow cytometry analysis for BrdU incorporation at 5560pM ligand concentration** 5560pM of each FGF2 isoform was used to determine relative mitogenic activity of each isoform with individual FGFR variants. FGFR1 IIIb, FGFR2 IIIb and FGFR3 IIIb were tested. X-axis indicates fluorescence corresponding to cells stained positively with anti-BrdU antibody. Numbers for each graph indicate percentage of cells incorporating BrdU.